AD ________________

Award Number: DAMD17-97-1-7234

TITLE: Role of cdc37 in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Lilia J. Stepanova
Jeffrey Harper

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010406 139

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (15 Jun 97 - 14 Jun 00)

4. TITLE AND SUBTITLE
Role of cdc37 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7234

6. AUTHOR(S)
Dr. Lilia J. Stepanova Jeffrey Harper

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

E-MAIL:
ls691035@mbcr.bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

p50^{Cdc37} functions in the establishment of protein kinase signaling pathways by functioning in complex with molecular chaperone Hsp90. Cdc37/Hsp90 complex plays a central role in the establishment of pathways that are directly implicated in cell cycle promotion and transformation. Cdc37 targets intrinsically unstable kinases to the complex with Hsp90, and this transient interaction of newly synthesized kinases with the complex is necessary for their stabilization and/or folding and further activation. We created and analyzed a transgenic mouse model where Cdc37 expression is driven by MMTV or PB promoter, directing the expression into breast or prostate epithelial cells, respectively. Our study show that Cdc37 is a first identified chaperone functioning as an oncogene, and is capable of cooperating with other oncogenes such as c-*myc, cyclin D1* and *neu* for transformation.

14. SUBJECT TERMS
Breast Cancer, Predoc Award

15. NUMBER OF PAGES
32

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

LS X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

LS X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

LS X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

LS N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 7/12/00
Date

AWARD NUMBER DAMD 17-97-1-7234

Role of Cdc37 in breast cancer

PI Lilia J. Stepanova

TABLE OF CONTENTS

INTRODUCTION

$p50^{Cdc37}$ functions in the establishment of protein kinase signaling pathways by functioning in complex with molecular chaperone Hsp90. Cdc37/Hsp90 complex plays a central role in the establishment of pathways that are directly implicated in cell cycle promotion and transformation. Cdc37 targets intrinsically unstable kinases to the complex with Hsp90, and this transient interaction of newly synthesized kinases with the complex is necessary for their stabilization and/or folding and further activation. We created and analyzed a transgenic mouse model where Cdc37 expression is driven by MMTV or PB promoter, directing the expression into breast or prostate epithelial cells, respectively. Our study show that Cdc37 is a first identified chaperone functioning as an oncogene, and is capable of cooperating with other oncogenes such as c-*myc, cyclin D1* and *neu* for transformation.

RESULTS AND DISCUSSION

1. Cdc37 is an oncogene.

We created two lines of transgenic mice expressing Cdc37 under control of MMTV promoter. Both lines develop tumors at about 18-22 months of age. This finding was presented in previous report.

2. Cdc37 cooperates with c-*myc* and *neu* oncogenes.

Cdc37 can cooperate with c-*myc* in transformation of multiple tissues including mammary and salivary epithelium and Leydig cells. Cdc37 can cooperate with neu oncogene by increasing the mitotic index of developing mammary tumors. These findings were presented in last year's report.

3. Cooperation of Cdc37 with cyclin D1.

Animals expressing both cyclin D1 and *CDC37* display evidence of mammary tumors at the age of 13 months, which is significant earlier than the animals with the similar genetic background expressing *CDC37* only. No animals expressing cyclin D1 or *CDC*37 alone developed tumors by 15 months of age in our experiment, indicating cooperative interaction of CDC37 with cyclin D1 in the transformation of the mammary gland.
Tumors developed by double transgenic animals appeared as rapidly dividing single mass adenocarcinomas. Majority of the animals developed one mammary tumor, and frequent metastasis to the lung were observed during pathological.

4. MMTV-Cdc37/MMTV-Ras crosses.

Originally we intended to characterize cooperation between Cdc37 and Ras oncogene. In the process of doing this we encountered technical problems which resulted in the abortion of this particular experiment. Partially it is due to the rapid development of tumors by these animals. By 3-4 months of age these animals develop multiple aggressive tumors and either die of this or should be sacrificed according to Animal Care Guidelines. Because of multiple tumors, females are not able to deliver more than 2 broods of pups, and their fertility is generally low. Expression of Ras oncogene in the mammary gland affects its function, and females are not able to lactate their pups because of the massive overt proliferation of the mammary epithelium. Pups of the transgenic females should be weaned by a foster mother. Moreover, MMTV-Ras transgenic males are infertile in 75% of animals. Due to these problems MMTV-Ras colonies of mice are extremely difficult to maintain, and, although these mice are available commercially through Jackson Labs, only few animals are available for distribution at any particular time. Because of the high demand for these animals and limited resources, we received only one MMTV-Ras transgenic female instead of many ordered. This female was crossed with non-

transgenic male, and it gave birth to one transgenic pup, which was male and later proved to be infertile. These two MMTV-Ras animals gave us multiple tumor samples which were used in biochemical analysis.

5. Summary

Detailed information on the results and discussion can be found in paper reprints in Appendices.

L. Stepanova, G. Yang, F. DeMayo, T.M. Wheeler, M. Finegold, T.C. Thompson, and J.W. Harper (2000). Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to induce prostatic hyperplasia. ***Oncogene*** 19, 2186-2193.

L. Stepanova, M.J. Finegold, F. DeMayo, E. Schmidt, and J.W. Harper (2000). The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene in Mice and Collaborates with both c-myc and cyclin D1 in Transformation of Multiple Tissues. ***Mol. Cell. Biol.*** 20, 4462-4473.

KEY RESEARCH ACCOMPLISHMENTS

- Discovery of the oncogenic properties of *CDC37*, first oncogene of chaperone class
- *CDC37* is able to cooperate with c-*myc* in transformation of multiple tissues
- *CDC37* is able to cooperate with *neu* in increasing the mitotic index of the mammary tumors
- *CDC37* is able to cooperate with cyclin D1 in transformation of mammary epithelia
- MMTV-*CDC37* animals have developmental defects, which are consistent with its positive role in promoting cell division
- PB-*CDC37* males develop neoplastic changes in prostate

REPORTABLE OUTCOMES

- Manuscripts:

L. Stepanova, G. Yang, F. DeMayo, T.M. Wheeler, M. Finegold, T.C. Thompson, and J.W. Harper (2000). Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to induce prostatic hyperplasia. ***Oncogene*** 19, 2186-2193.

L. Stepanova, M.J. Finegold, F. DeMayo, E. Schmidt, and J.W. Harper (2000). The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene in Mice and Collaborates with both c-myc and cyclin D1 in Transformation of Multiple Tissues. ***Mol. Cell. Biol.*** 20, 4462-4473.

- Abstracts and presentations

1998: 51st Annual Symposium on Fundamental Cancer Research: Molecular Targets for Cancer Therapy and Prevention, Houston, TX. Altered Proliferation and Tumorigenesis in MMTV-Cdc37 transgenic Mice and Potential Collaboration with Myc in Induction of Salivary Gland Tumors.

1999: 28th National Student Research Forum, Galveston, TX
Oncoprotein Kinase Chaperone Cdc37 is a New Oncogene Able to Cooperate with Other Oncogenes

1999: 1999 Biochemistry and Molecular Biology Meeting/ ASBMB Satellite Meeting, San Francisco, CA
Cdc37 is a First Protein Chaperone with Properties of an Oncogene

1999: 8th Annual Oncology Research Seminar, Houston, TX
Cdc37 is a Chaperone and a New Oncogene.

2000: 5th Cold Spring Laboratory Meeting on the Cell Cycle. Cold Spring Harbor, NY.
Cdc37 Expression Causes Transformation in Transgenic Mice.

2000: Era of Hope Meeting, Atlanta, GA
Protein Kinase Chaperone Cdc37 Causes Transformation of the Mammary Epithelium in Transgenic Mice and Can Cooperate with Other Oncogenes in Transformation of Multiple Tissues.

- Licenses

Licenses with StressGen company (Canada) for a plasmid expressing Cdc37 and antibodies against Cdc37

- Degrees awarded:

4 January, 2000 - PhD in Biochemistry and Molecular Biology (Baylor College of Medicine, Houston, TX) awarded to Lilia Stepanova.

- Animal models:

MMTV-Cdc37 and PB-Cdc37 transgenic mice are placed with The Jackson Laboratory, an independent non-profit mammalian research laboratory. The colonies are currently under development.

CONCLUSIONS

Our study show for the first time that Cdc37 is an oncogene and is able to cooperate with other oncogenes in transformation of multiple tissues. This is the first implication of the chaperone pathway in abnormal proliferation in mammalian organism.

The results were published in two high-ranking journals and presented on several national meetings and conferences.

The published papers are expected to generate a significant interest in the role of Cdc37 in development of human cancers, and search for mutations affecting normal Cdc37 functions.

This study lead to the development of several key research components such as plasmids, antibodies and transgenic animals that are available to other researchers interested in continuing research on Cdc37. A doctorate degree in biochemistry and molecular biology has been awarded in 2000 to Lilia Stepanova, PI in this research.

APPENDICES

L. Stepanova, G. Yang, F. DeMayo, T.M. Wheeler, M. Finegold, T.C. Thompson, and J.W. Harper (2000). Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to induce prostatic hyperplasia. ***Oncogene*** 19, 2186-2193.

L. Stepanova, M.J. Finegold, F. DeMayo, E. Schmidt, and J.W. Harper (2000). The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene in Mice and Collaborates with both c-myc and cyclin D1 in Transformation of Multiple Tissues. ***Mol. Cell. Biol.*** 20, 4462-4473.

Molecular and Cellular Biology, June 2000, p. 4462–4473
0270-7306/00/$04.00+0 Vol. 20, No. 12

The Oncoprotein Kinase Chaperone *CDC37* Functions as an Oncogene in Mice and Collaborates with Both c-*myc* and Cyclin D1 in Transformation of Multiple Tissues

LILIA STEPANOVA,[1]† MILTON FINEGOLD,[2] FRANCO DEMAYO,[3] EMMETT V. SCHMIDT,[4] AND J. WADE HARPER[1]*

Verna and Marrs McLean Department of Biochemistry and Molecular Biology,[1] Department of Pathology,[2] and Department of Molecular and Cellular Biology,[3] Baylor College of Medicine, Houston, Texas 77030, and The MGH Cancer Center, Department of Tumor Biology, Charlestown, Massachusetts 02129[4]

Received 5 November 1999/Returned for modification 28 December 1999/Accepted 15 March 2000

CDC37 encodes a 50-kDa protein that targets intrinsically unstable oncoprotein kinases including Cdk4, Raf-1, and v-src to the molecular chaperone Hsp90, an interaction that is thought to be important for the establishment of signaling pathways. CDC37 is required for proliferation in budding yeast and is coexpressed with cyclin D1 in proliferative zones during mouse development, a finding consistent with a positive role in cell proliferation. CDC37 expression may not only be required to support proliferation in cells that are developmentally programmed to proliferate but may also be required in cells that are inappropriately induced to initiate proliferation by oncogenes. Here we report that mouse mammary tumor virus (MMTV)-CDC37 transgenic mice develop mammary gland tumors at a rate comparable to that observed previously in MMTV-cyclin D1 mice. Moreover, CDC37 was found to collaborate with MMTV–c-myc in the transformation of multiple tissues, including mammary and salivary glands in females and testis in males, and also collaborates with cyclin D1 to transform the female mammary gland. These data indicate that CDC37 can function as an oncogene in mice and suggests that the establishment of protein kinase pathways mediated by Cdc37-Hsp90 can be a rate-limiting event in epithelial cell transformation.

Extracellular signals act to coordinate proliferation during the first gap (G_1) phase of the cell division cycle. These signals typically act through receptor tyrosine kinases to activate protein kinase signaling pathways that direct the expression of genes required for proliferation. Recent studies have implicated components of the *ras* pathway in regulating the expression of D-type cyclins, a central component of mitogen-dependent cell cycle entry (1, 41). Ras activation leads to engagement of the Raf/MEK/MAPK pathway (47, 60, 65, 70, 72), and each of these components is necessary and sufficient to induce cyclin D expression (1, 2, 21, 27, 41, 69). D-type cyclins are essential activator subunits of Cdk4 and Cdk6, and holoenzyme complexes of these kinases have been implicated in cell cycle entry through multiple mechanisms. Cyclin D-Cdk4 complexes directly phosphorylate retinoblastoma protein (Rb) and initiate inactivation of its growth suppressor function (9, 12, 20, 34, 36). In addition, cyclin D-Cdk4 complexes may contribute to the activation of cyclin E-Cdk2 by titrating the Cdk inhibitor $p27^{KIP1}$ from Cdk2 complexes (8, 19, 35, 45, 46, 55). Consistent with the central role of cyclin D in *ras*-dependent proliferation is the finding that Cdk4 inhibitors of the p16 class can inhibit *ras*-mediated proliferation in an Rb-dependent manner (30, 37, 41, 52).

The assembly of cyclin D-Cdk4 complexes is complex and appears to involve multiple steps, including a mitogen-dependent step (7, 8, 24, 34, 36). Previously, we cloned a mammalian homolog of the budding yeast and avian *CDC37* gene (4, 15) and demonstrated that $p50^{Cdc37}$ binds to Cdk4 and Cdk6 but not to Cdc2 and Cdk2 (58). In budding yeast, *CDC37* is an essential gene and is required for formation of Cdc28-Cln complexes through an unknown mechanism (14). We and others have demonstrated that mammalian Cdc37 assembles with Cdk4 in high-molecular-weight complexes that also contain the molecular chaperone Hsp90 (11, 25, 58). Molecular analysis revealed that the *CDC37* gene encodes the Hsp90-associated p50 protein (42, 58), previously seen in complexes with v-*src* (5, 6, 18, 66) and Raf (57) but whose identity was unknown. Cdc37 associates with Hsp90 independently of protein kinases and appears to function at least in part as a protein kinase-targeting subunit of Hsp90 (58). Genetic and biochemical data in several systems suggest that particular protein kinases are intrinsically unstable and their association with the Cdc37-Hsp90 chaperone is important for folding and/or activation of the targeted kinase (10, 14, 16, 38, 58, 71). Once Cdk4 is stabilized by the Cdc37 complex, it is released in a step that is not characterized and can then assemble with either inhibitors such as p16 or with cyclin D. Assembly with cyclin D requires a member of the p21 class of Cdk inhibitors, possibly in addition to a mitogen-dependent step (7, 24, 40).

CDC37 is expressed primarily in proliferative zones during embryonic development and in adult tissues, and its pattern of expression closely corresponded to that of cyclin D1 (58). Interestingly, *CDC37* is not expressed in several adult tissues including virgin mammary duct epithelial cells but, like cyclin D1, is induced during pregnancy, consistent with a positive role in proliferation (58). These data, coupled with the fact that *CDC37* is required for proliferation in budding yeast and *Drosophila* cells (10), suggest that *CDC37* expression may be required to support proliferation in those cells that are developmentally programmed to proliferate but may also be required in those cells that are inappropriately induced to initiate pro-

* Corresponding author. Mailing address: Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-6993. Fax: (713) 796-9438. E-mail: jharper@bcm.tmc.edu.

† Present address: Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105.

liferation by oncogenes. If this were the case, then *CDC37* would be predicted to collaborate with transforming oncogenes. Standard tissue culture-based assays that measure oncogenic collaboration employ fibroblasts which already express high levels of Cdc37 (58), suggesting that this approach may not reveal the collaborative potential of Cdc37. Therefore, we sought to examine the effects of Cdc37 in vivo by targeting its expression to cells in the mammary gland and other tissues where it is normally not present in the adult animal. Mouse mammary tumor virus (MMTV)-*CDC37* transgenic mice were found to develop mammary gland tumors at a rate comparable to that observed in MMTV-cyclin D1 mice. Moreover, *CDC37* was found to collaborate with MMTV–c-*myc* in the transformation of multiple tissues, including mammary and salivary glands in females and testis in males, and with cyclin D1 in the mammary gland. In a parallel study (58a), we found that Cdc37 is absent from normal human prostate but is abundant in human prostate cancer. Interestingly, selective expression of *CDC37* in the prostate leads to hyperplasia in transgenic mice (58a). Taken together, these data indicate that Cdc37 can function as an oncogene in mice and suggest that the establishment of protein kinase pathways mediated by Cdc37-Hsp90 can be a rate-limiting event in epithelial cell transformation.

MATERIALS AND METHODS

Generation of transgenic mice. An MMTV-*CDC37* transgene was generated by cloning a *Xho*I fragment containing the 1.6-kb mouse *CDC37* open reading frame (ORF) into a plasmid containing an MMTV promoter, beta-globin splice sequences, and bGH polyadenylation sequences. The 4.63-kb transgene fragment was released from the plasmid by digesting with *Not*I/*Kpn*I and then purified. Transgene DNA was microinjected into male pronuclei of B6D2F1 mouse embryos in the Baylor College of Medicine transgenic core facility. Resulting pups were screened by Southern analysis of genomic DNA isolated from mouse tails digested with *Bam*HI. To establish lines of transgenic mice, founders were continuously mated with ICR mice. Nontransgenic littermates of heterozygous parents were used as controls. MMTV-*CDC37* heterozygous females were mated with MMTV–c-*myc* (Charles River Laboratory) or MMTV-cyclin D1 homozygous transgenic males (64). Both MMTV–c-*myc* and MMTV-cyclin D1 mice were on a inbred FVB genetic background. Resulting progeny carried either both transgenes (c-*myc*+*CDC37* or cyclin D1+*CDC37*) or a single transgene (c-*myc* or cyclin D1). Both groups of animals were monitored for tumor formation for comparison. For nontransgenic controls, MMTV-*CDC37* heterozygous females were crossed with nontransgenic FVB males. The copy number was determined by quantitative Southern blotting of mixtures of tail DNA from nontransgenic and transgenic mice, followed by phosphorimager analysis. This analysis gave 8 and 5 copies for the MMTV-Cdc37.1 and MMTV-Cdc37.2 lines, respectively.

Northern analysis. Total RNA was prepared from mouse tissues, separated on an 1% agarose gel, transferred to Hybond N+ (Amersham) membrane, and blotted with a ^{32}P-labeled *CDC37* cDNA probe to detect endogenous and transgene derived transcripts, or a 5′+3′ probe consisting of rabbit beta-globin splice site sequences and bovine polyadenylation signal DNA, which was used to detect only exogenous *CDC37* transcripts. Blots were stripped and reprobed with a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe to control for RNA levels. In some experiments, blots were also probed with a c-*myc* cDNA probe provided by M. Cole.

Histology and immunohistochemistry. For histological analysis, mouse tissues were excised and fixed in 4% formaldehyde–phosphate-buffered saline PBS overnight at 4°C prior to being embedded in paraffin. Embedded tissues were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). For immunohistochemistry, 5-μm sections were stained with rabbit polyclonal affinity-purified Cdc37 antibodies or with anti-c-*myc* antibodies (NeoMarkers) as described previously (58).

Western blot analysis. Frozen tumor specimens were used for preparation of protein lysates by homogenization in NP-40 buffer (58), followed by centrifugation and determination of protein concentration by Bradford assays. For Western blotting, 200 μg of extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% PAGE gel and then transferred to nitrocellulose. Blotting was performed using polyclonal Cdc37 antibodies (58), Cdk4, Erk1 and Erk2, and c-*myc* antibodies from Santa Cruz or anti-phospho-ERK from New England Biolabs. Detection was accomplished by using horseradish peroxidase-conjugated secondary antibodies in combination with enhanced chemiluminescence (Amersham).

Whole-mount analysis. Inguinal fat pads were excised from the animals, spread on a glass surface and fixed in 10% formalin for 10 to 12 h, and washed in acetone for 48 h, followed by washing in 100 and 95% ethanol (EtOH) for 1 h each. Tissues were stained with hematoxylin for 12 h (0.3% [wt/vol] hematoxylin and 0.34% [wt/vol] $FeCl$ in 0.06 N HCl–80% EtOH). Stained tissues were washed for 1 h in distilled water and increasing concentrations of EtOH (70 to 100%) and finally in xylene. Tissues were stored in glass vials, covered with methyl salicylate.

RESULTS

MMTV-*CDC37* transgenic mice. To assess the possible role of *CDC37* in promoting neoplastic transformation, transgenic mice expressing mouse *CDC37* under the control of the MMTV promoter (Fig. 1A) were generated. Two transgenic founders (Fig. 1B) were produced which transmitted the transgene to their progeny in a Mendelian fashion. Lines of transgenic animals (MMTV-*CDC37*.1 and MMTV-*CDC37*.2) were established by mating each founder with outbred ICR mice. Quantitative analysis of copy number revealed eight and five transgenes, respectively, for MMTV-Cdc37.1 and MMTV-Cdc37.2 strains (see Materials and Methods). The expression of Cdc37 was examined by Northern blotting, immunoblotting, and immunofluorescence, with an emphasis on tissues known to express MMTV-driven transgenes. *CDC37* mRNA was high in the lacrimal, mammary, and salivary glands, uterus, and testis, using both the *CDC37* cDNA (Fig. 1C) and transgene-specific regulatory sequences (5′+3′) (data not shown) as probes, compared to the low levels found in these tissues in nontransgenic animals. The levels of mRNA in the MMTV-*CDC37*.2 strain was ~50% of those in the MMTV-*CDC37*.1 line (data not shown), a finding consistent with the lower copy number. Consistent with this, immunoblot analysis revealed that the Cdc37 protein was undetectable in extracts from normal salivary and virgin mammary glands but was readily detectable in extracts from transgenic mice (Fig. 1D). We previously reported that Cdc37 sometimes migrates as a doublet by SDS-PAGE (58). In normal virgin mammary gland, the more slowly migrating form of Cdc37 is predominant, while the more rapidly migrating form is predominant in salivary tissue. Cdc37 is a phosphoprotein (7), and we have shown that it is phosphorylated by casein kinase in vitro at sites that are also modified in vivo (data not shown). Thus, these isoforms may reflect differential phosphorylation in different tissues. To quantitatively address Cdc37 levels relative to those found normally in cycling cells, we examined Cdc37 protein by immunofluorescence and compared the levels with that found in sites of known Cdc37 expression in vivo. Transgenic Cdc37 was found in the majority of epithelial cells in the salivary gland (Fig. 2A) and Leydig cells in the testis (see Fig. 5) but was not detected in these cell types in nontransgenic animals. In the virgin mammary gland, Cdc37 was present in ~30% of ductal epithelial cells (data not shown). Although *CDC37* mRNA appears to be quite abundant, when examined at the single cell level, the levels of Cdc37 protein in all three tissues examined was similar to that found in proliferative cells in the intestine and in cycling BALB/c fibroblasts in culture (Fig. 2C). It is possible that translational and/or posttranslational events may control the total level of Cdc37 achievable in these tissues.

Ectopic expression of *CDC37* in the mouse breast leads to transformation. MMTV-*CDC37* lines and control littermates were maintained as breeding colonies and monitored for developmental and transformation phenotypes for up to 2 years. Transgenic animals appeared normal at birth, and their growth was indistinguishable from their nontransgenic littermates. Their reproduction, number of pups per litter, and lactation in females were normal, although promiscuous male breast development was detected (see below).

Malignant transformation of the mammary gland or other

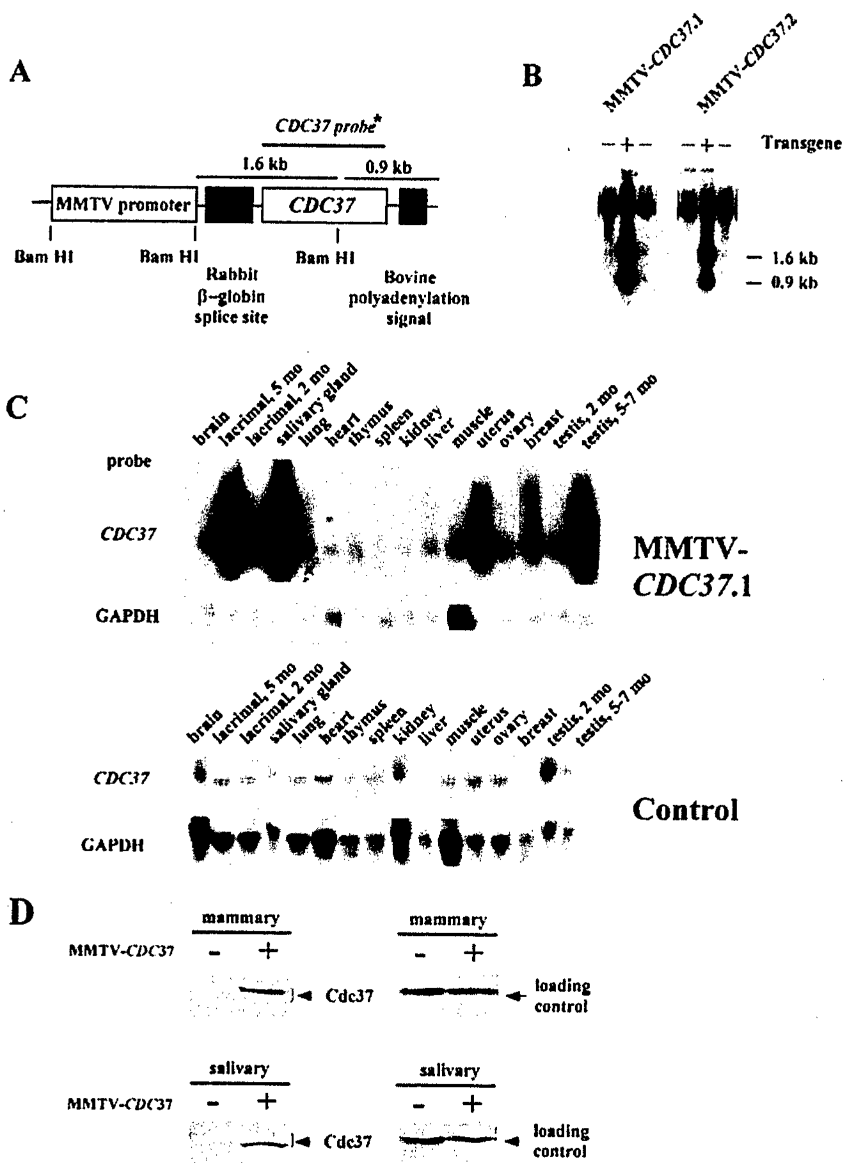


FIG. 1. Characterization of MMTV-*CDC37* transgene expression. (A) Structure of the construct used to generate MMTV-*CDC37* mice (see Materials and Methods for details). (B) Southern blot analysis of MMTV-*CDC37*.1 and MMTV-*CDC37*.2 transgenic lines. Tail DNA was digested with *Bam*HI prior to Southern analysis with the *CDC37* cDNA. The 1.6-kb band corresponds to the construct fragment containing rabbit β-globin splice site, and the 0.9-kb bands represent fragments containing bovine polyadenylation signal (see panel A). +, Mice containing the transgene; −, mice lacking the transgene. (C) Northern blot analysis of *CDC37* expression in tissues derived from transgenic and control animals. Total RNA was hybridized with the *CDC37* cDNA which detects both endogenous *CDC37* and the transgene derived message. The GAPDH probe (GAPDH ORF) is used as a loading control. Muscle tissue has intrinsically higher levels of GAPDH mRNA. (D) Immunoblot analysis of Cdc37 in nontransgenic and MMTV-Cdc37.1 mice. Tissue extracts (100 μg) from the indicated tissues were separated by SDS-PAGE and blotted with affinity-purified anti-Cdc37 antibodies. A nonspecific cross-reacting band observed with monoclonal antibody 9E10 was used as a loading control.

organs was not observed during first 1.5 years of life in *CDC37* transgenic animals. However, as MMTV-*CDC37* animals approached 18 months of age, a significant fraction of animals from both lines developed proliferative disorders, including mammary tumors and lymphomas (Table 1; Fig. 3 and 4A). Histopathological analysis indicated that mammary tumors were adenocarinomas and adenosquamous carcinomas (Fig. 3). By 22 months of age, 100% of MMTV-*CDC37*.1 females had developed tumors in the mammary or lymphoid compartments (Fig. 3A and 4A; Table 1). Mammary tumors arose as singular persistent masses adjacent to normal mammary epithelium. Mitotic figures were rare, indicative of slow-growing carcinomas. Histopathological examination also revealed enlarged nuclei and frequent keratin deposits which are indicative of squamous differentiation (Fig. 4A). Necrotic and apoptotic changes were minimal. Immunohistochemistry revealed Cdc37 protein expression in a large fraction of tumor cells (Fig. 4A). Lymphomas in transgenic females usually manifested themselves as an extreme weakness of the animals and obvious enlargement of the lymph nodes. Two cases of lymphomas were discovered in animals that already had developed mammary adenosquamous carcinomas. All lymphomas exhibited very low mitotic activity (data not shown), which could explain the slow progression of disease. Twenty animals of the MMTV-*CDC37*.2 line were autopsied at 17 months of age. Nine animals displayed evidence of proliferative disorders (Ta-

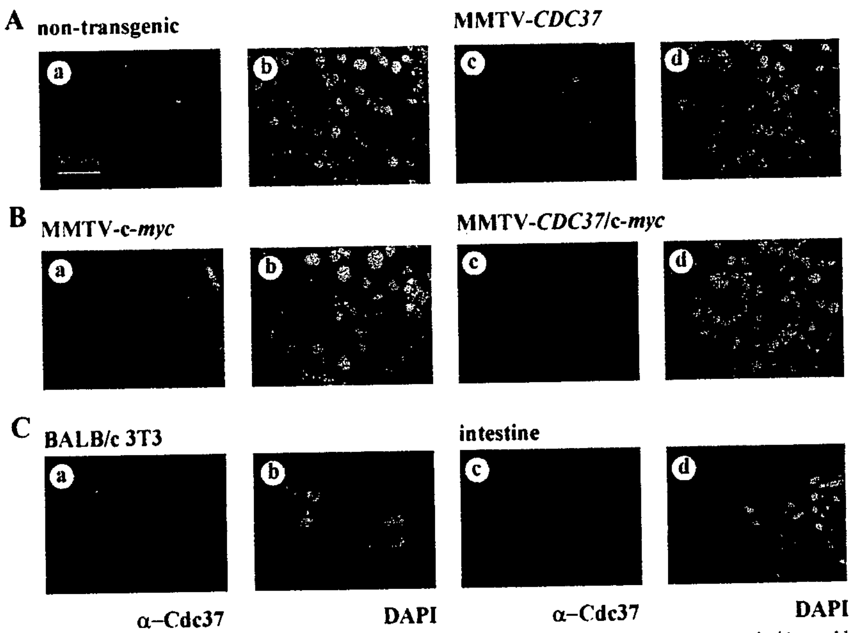


FIG. 2. Analysis of Cdc37 expression by immunofluorescence. (A and B) Salivary gland tissue sections from nontransgenic (Aa and b), MMTV-*CDC37* (Ac and d), MMTV–c-*myc* (Ba and b), and MMTV–*CDC37*/c-*myc* (Bc and d) mice were stained with anti-Cdc37 antibodies and visualized with secondary antibodies labeled with fluorescein isothiocyanate (FITC). Nuclei were visualized with DAPI (4′,6′-diamidino-2-phenylindole). (C) BALB/c 3T3 cells (a and b) or intestinal sections from nontransgenic mice (c and d) were probed with anti-Cdc37 and nuclei identified by DAPI. In the intestine, Cdc37 expression is limited to a narrow band of proliferating cells (58). The same exposures were used for all figures.

ble 1), primarily mammary adenosquamous carcinomas and lymphomas, although one case of sarcoma was found. As in the first line examined, all tumors displayed a low mitotic index with little evidence of apoptosis. Nontransgenic control animals were subjected to a detailed pathological analysis either in parallel with *CDC37* transgenic animals or at 17 to 22 months of age. No evidence of proliferative disturbances was found in nontransgenic animals (Fig. 3A and Table 1).

***CDC37* cooperates with c-*myc* in induction of mammary tumors in breeding females.** *CDC37* is expressed in proliferative zones in adult tissues and is coexpressed with cyclin D1 in several tissues, but it is absent in many differentiated cell types, including many epithelial cell types (58). We therefore hypothesized that *CDC37* expression might be required to support transformation by oncogenic pathways. In this case, we would predict that inappropriate *CDC37* expression might promote proliferative events dependent on oncogenic pathways.

To test this, we crossed MMTV-*CDC37* heterozygous females with MMTV–c-*myc* and MMTV-cyclin D1 homozygous males. To control for differences in genetic backgrounds, we monitored heterozygous c-*myc* and cyclin D1 littermates alongside the double transgenics. Previously, it was shown that multiple rounds of pregnancy and lactation are able to promote expression of the c-*myc* transgene and accelerate tumorigenesis (56). We evaluated the influence of the level of expression of the transgene on tumorigenesis by dividing single and double transgenic females into two groups: one was kept virgin, and the other was kept in the presence of breeder males. Both lines of *CDC37*-expressing animals were used for these experiments. The approximately equal number of double transgenic females carried either MMTV-*CDC37*.1 or MMTV-*CDC37*.2 in combination with c-*myc* transgene. No differences between the two lines were observed in the kinetics of tumor appearance and tumor specificity in either breeding or virgin double transgenic females, and therefore the data for two lines were pooled together (Fig. 3A).

Tumors were observed in breeding MMTV–c-*myc* females as early as 3 months of age and 50% of females had developed tumors by 250 days of age in this genetic background (Fig. 3A and 4B). In contrast, breeding females carrying both c-*myc* and *CDC37* transgenes developed tumors with accelerated kinetics, and 50% of females developed tumors by the age of 115 days (Fig. 3A and 4C). All tumors developed by breeding females were mammary ductal and alveolar adenocarcinomas (Fig. 3B). In addition to the acceleration of tumor incidence, we also observed a dramatic increase in the number of tumors/animal

TABLE 1. Neoplasms found in transgenic females carrying the MMTV-*CDC37* transgene

Pathology	Line	% Mice affected (no./total)
Mammary ductal adeno-squamous carcinoma	MMTV-*CDC37*.1	60 (6/10)
	MMTV-*CDC37*.2	20 (4/20)
Lymphoma	MMTV-*CDC37*.1	50 (5/10)
	MMTV-*CDC37*.2	20 (4/20)
Mammary ductal adeno-carcinoma	MMTV-*CDC37*.1	10 (1/10)
	MMTV-*CDC37*.2	0 (0/20)
Sarcoma	MMTV-*CDC37*.1	0 (0/10)
	MMTV-*CDC37*.2	5 (1/20)
Total affected	MMTV-*CDC37*.1[a]	100 (10/10)
	MMTV-*CDC37*.2[b]	45 (9/20)
	Nontransgenic[c]	0 (0/30)

[a] Tumors occurred between 18 and 22 months of age.
[b] All animals were sacrificed and analyzed at 17 months of age without outward signs of transformation.
[c] Animals were examined in parallel with MMTV-*CDC37* mice at 17 to 22 months of age.

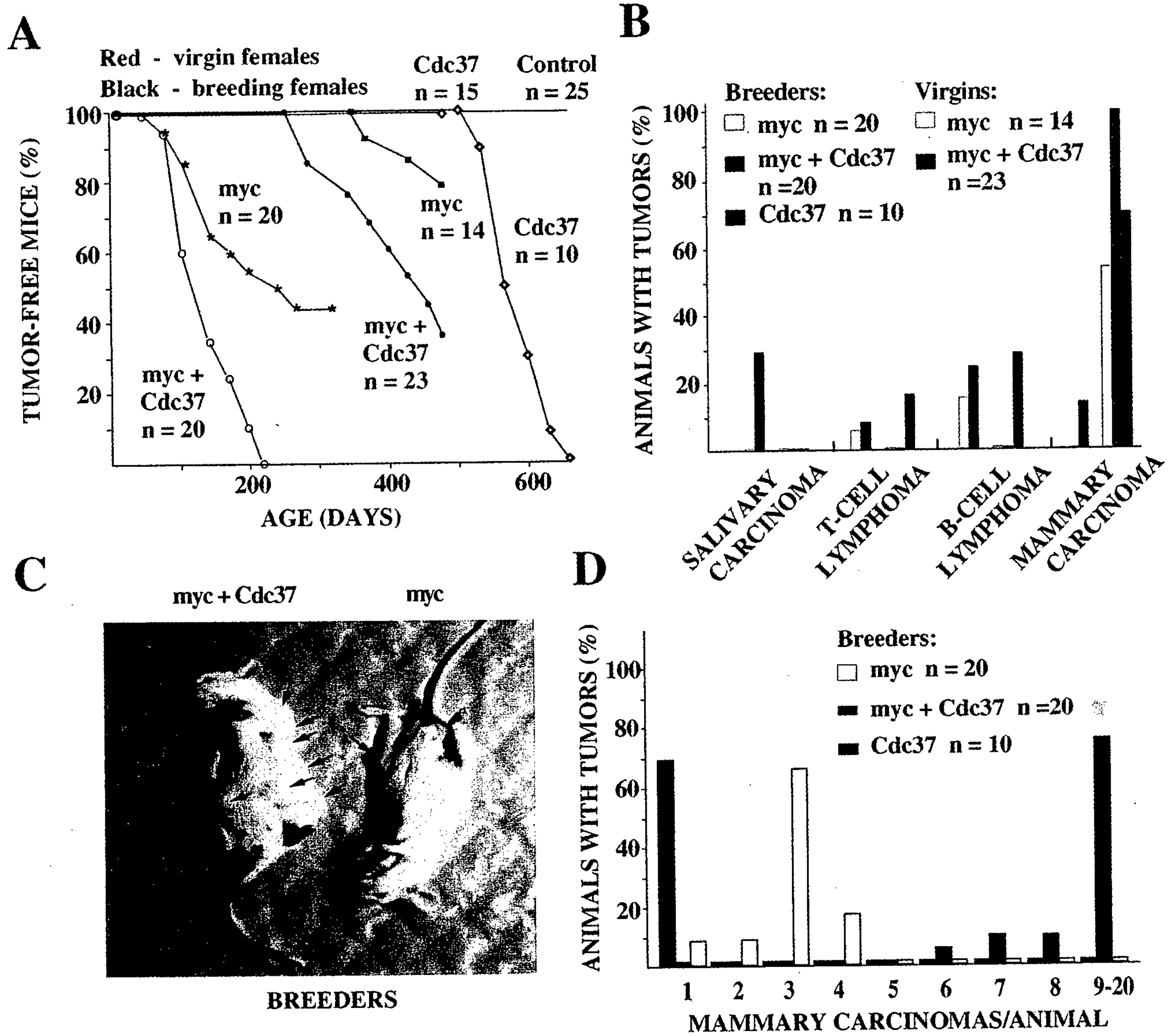


FIG. 3. MMTV-*CDC37* facilitates transformation of the mouse mammary epithelium and collaborates with c-*myc* to transform multiple tissues. (A) Quantitation of incidence of proliferative disorders. Tumor-free animals from breeding females are shown in black, while the tumor incidence in virgin animals is shown in red. n, number of animals in each group. Data shown for Cdc37 mice were from the MMTV-*CDC37*.1 line. Breeding and virgin MMTV–*CDC37*/c-*myc* females bore either MMTV-*CDC37*.1 or MMTV-*CDC37*.2 transgenes. (B) Types of tumors developed by virgin or breeding MMTV-*CDC37*, MMTV–c-*myc*, and double transgenic MMTV–*CDC37*/c-*myc* mice. The percentage of the animals developing each type of tumor from panel A is shown. Some of the animals developed more than one type of malignancy. The ages of breeding animals were as follows: MMTV-*CDC37*, 17 to 22 months; MMTV–c-*myc*, 3 to 12 months; and MMTV–c-*myc*/*CDC37*, 3 to 7 months. The ages of virgin animals were as follows: MMTV–c-*myc*, 12 to 16 months; and MMTV–c-*myc*/*CDC37*, 9 to 16 months. (C) Gross appearance of the breeding females expressing either MMTV–c-*myc* (right) or MMTV–*CDC37*/c-*myc* (left). The double transgenic females develop more tumors per animal than do single c-*myc* transgenics. The additional tumors, which were not visible by gross examination, were detected by detailed histopathological analysis. (D) Quantitation of tumor number per animal. The percentage of animals developing a given number of mammary adenocarcinomas is shown. MMTV-*CDC37* animals developed only one tumor per animal. c-*myc*-expressing animals developed from 1 to 4 tumors/animal, while the majority of the double transgenics had between 9 and 20 tumors/animal. The number of tumors was estimated by counting foci on sections from fixed preparations of all mammary glands. The ages of animals are given in panel B.

(Fig. 3C and D). This included both an increase in the number of glands affected as well as the number of tumors/gland (Fig. 3D). While MMTV–c-*myc* animals rarely had all of the glands affected, virtually all of the double transgenic animals were affected in every gland (Fig. 3C). While MMTV–c-*myc* females had on average three tumors per animal, MMTV–*CDC37*/c-*myc* approached 20 tumors per animal, on average (Fig. 3D). In many cases, the tumor masses were so abundant it prevented an exact determination of the number of tumor foci. On sections of both MMTV–*CDC37*/c-*myc* and MMTV–c-*myc* mammary glands all transitions from normal to transformed epithelium could be seen, including multiple areas of hyperplasia.

Altered tissue specificity of transformation in nonbreeding MMTV–*CDC37*/c-*myc* females. *CDC37* is normally not expressed in virgin mammary epithelium but is induced during pregnancy. c-*myc* has been shown to induce mammary transformation in virgin mice in some genetic backgrounds, although the extent of transformation is much lower than was observed with multiple pregnancies. To examine whether

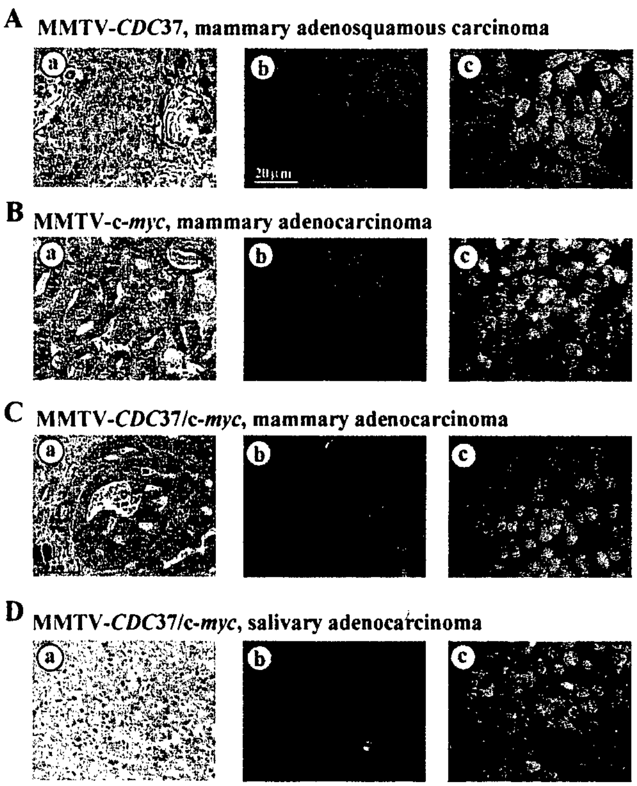


FIG. 4. Phenotypic analysis of tumors developed by MMTV-*CDC37* transgenic mice. (Aa) Ductal adenosquamous carcinoma of the mammary gland derived from an MMTV-*CDC37* mouse was stained with H&E. Arrows indicate squamous differentiation. (Ab and c) Adjacent tumor sections from Aa were stained with anti-Cdc37 antibodies and visualized with FITC (b), while nuclei were visualized with DAPI. H&E, ×400 magnification. Immunofluorescence, ×1,000 magnification. (B) Same as panel A except that the tumor was a mammary adenocarcinoma from an MMTV–*c-myc* mouse. (C) Same as panel A except that the tumor was a mammary adenocarcinoma from an MMTV–*CDC37*/*c-myc* mouse. (D) Same as panel A except that the tumor was a salivary gland adenocarcinoma from an MMTV–*CDC37*/*c-myc* mouse.

CDC37 can collaborate with c-*myc* in the absence of hormonal stimulation, we examined females maintained in the virgin state. In our strain background, MMTV–c-*myc* virgin females typically incurred B-cell lymphomas as opposed to mammary carcinomas (Fig. 3B). The kinetics of tumor development were very slow, and only 25% of females developed tumors by the age 500 days. In contrast, the kinetics of tumor incidence in double transgenics generated from both *CDC37* lines were substantially accelerated (Fig. 3A). Relative to single c-*myc* transgenics, the spectrum of tumors was much wider (Fig. 3B), including both T- and B-cell lymphomas, as well as mammary and salivary gland adenocarcinomas. In double transgenic females, a prevalent tumor type was salivary adenocarcinoma (Fig. 3B). Salivary tumors from MMTV–*CDC37*/c-*myc* animals contain readily detectable Cdc37 (Fig. 4D) and c-*myc* (data not shown). This tumor type has never been reported in c-*myc*-expressing animals, although c-*myc* expression is readily observed in the salivary of phenotypically normal salivary glands in MMTV–c-*myc* mice (see Fig. 6B). Adenocarcinomas found in double transgenics appeared to be fast growing, with many mitotic figures (Fig. 4D). Taken together, these data indicate that MMTV-*CDC37* can alter the rates and extent of transformation in both breeding and nonbreeding MMTV–c-*myc* mice and can also alter the specificity of transformation.

Testicular hyperplasia and transformation in MMTV–*CDC37*/c-*myc* males. MMTV–c-*myc*-expressing males are typically free of proliferative disorders (58). Therefore, we were surprised to find evidence of both overt Leydig cell tumors and testicular hyperplasia in double transgenic males (Fig. 5). Cdc37 is normally not detectable in the testis of an adult mouse but is readily apparent in Leydig cells in MMTV-*CDC37* mice (Fig. 5D). Leydig cell tumors were observed in MMTV–*CDC37*/c-*myc* male mice at as young as 10 months (Fig. 5A, G). One of the four tumor-bearing animals had two distinct Leydig cell tumors, one in each testis. At an age of ~400 days, about two-thirds of all apparently unaffected males were sacrificed, and their testes were subjected to detailed histological analysis. A significant fraction (75%) of double transgenic males displayed Leydig cell hyperplasia (Fig. 5F), a possible precursor to overt transformation. In contrast, only about 20% of MMTV–c-*myc* males displayed modest Leydig cell hyperplasia (Fig. 5B, C and E). Nontransgenic and MMTV-Cdc37 males did not display any hyperplasia.

Biochemical analysis of tumors derived from breeding MMTV–c-*myc* and MMTV–*CDC37*/c-*myc* transgenic females. To begin to address how *CDC37* and c-*myc* collaborate in transformation, we examined the levels of several protein kinases as well as c-*myc* in mammary carcinomas from MMTV–*CDC37*/c-*myc* and MMTV–c-*myc* animals (Fig. 6A). As a control, we also examined the levels of proteins in mammary tumors derived from an MMTV-*ras* mouse (59). As expected, Cdk4 levels were increased in tumors expressing MMTV-*CDC37* (Fig. 6A, lanes 3 to 6), relative to that found with MMTV–c-*myc* alone (lanes 7 to 10), as were the Erk1 levels. We also found that activated Erk levels were higher in MMTV–*CDC37*/c-*myc* mice than in MMTV–c-*myc* mice (Fig. 6A). Unexpectedly, we found that c-*myc* levels were also increased in the presence of MMTV-*CDC37* compared to animals expressing only MMTV–c-*myc* (Fig. 6A). The observed differences in protein levels cannot be explained by the increased number of dividing cells, since no significant difference was observed in the mitotic index of these tumors (data not shown). One explanation for increased c-*myc* abundance is that Cdc37 can affect expression from the MMTV promoter, thereby causing an indirect increase in c-*myc* levels. Analysis of c-*myc* mRNA in tissues derived from MMTV–c-*myc* and MMTV–c-*myc*/*CDC37* mice, however, revealed similar levels of c-*myc* mRNA (Fig. 6B). Thus, Cdc37 does not indirectly influence c-*myc* expression from the MMTV-transgene promoter. An alternative explanation is that Cdc37 expression causes an alteration in the population of cells expressing c-*myc*. To test this, we examined c-*myc* expression in sections containing phenotypically normal tissue from various tissues. c-*myc* staining was not detected in nontransgenic animals (Fig. 6C) but was evident in the cytoplasm of all epithelial cells in the salivary and mammary glands from MMTV–c-*myc* mice (Fig. 6C and data not shown). The presence of Cdc37 had no discernible effect on the levels or extent of c-*myc* expression (Fig. 2B and 6C), ruling out increased numbers of c-*myc*-positive cells as an explanation for the observed increase in c-Myc protein levels. Tumors derived from MMTV-*ras* and MMTV–c-*myc* mice contained primarily the more slowly migrating Cdc37 isoform, while MMTV–*CDC37*/c-*myc* tumors contain both Cdc37 isoforms (Fig. 6A).

***CDC37* cooperates with cyclin D1 in transformation of the mammary epithelium.** To further test the effect of simultaneous expression of *CDC37* with other oncogenes, we created transgenic animals expressing both *CDC37* and cyclin D1 under control of the MMTV promoter. Previous studies have demonstrated that MMTV-cyclin D1 mice develop mammary gland adenocarcinomas with an average age of onset of 534 days (64). In the genetic background of our study, no prolif-

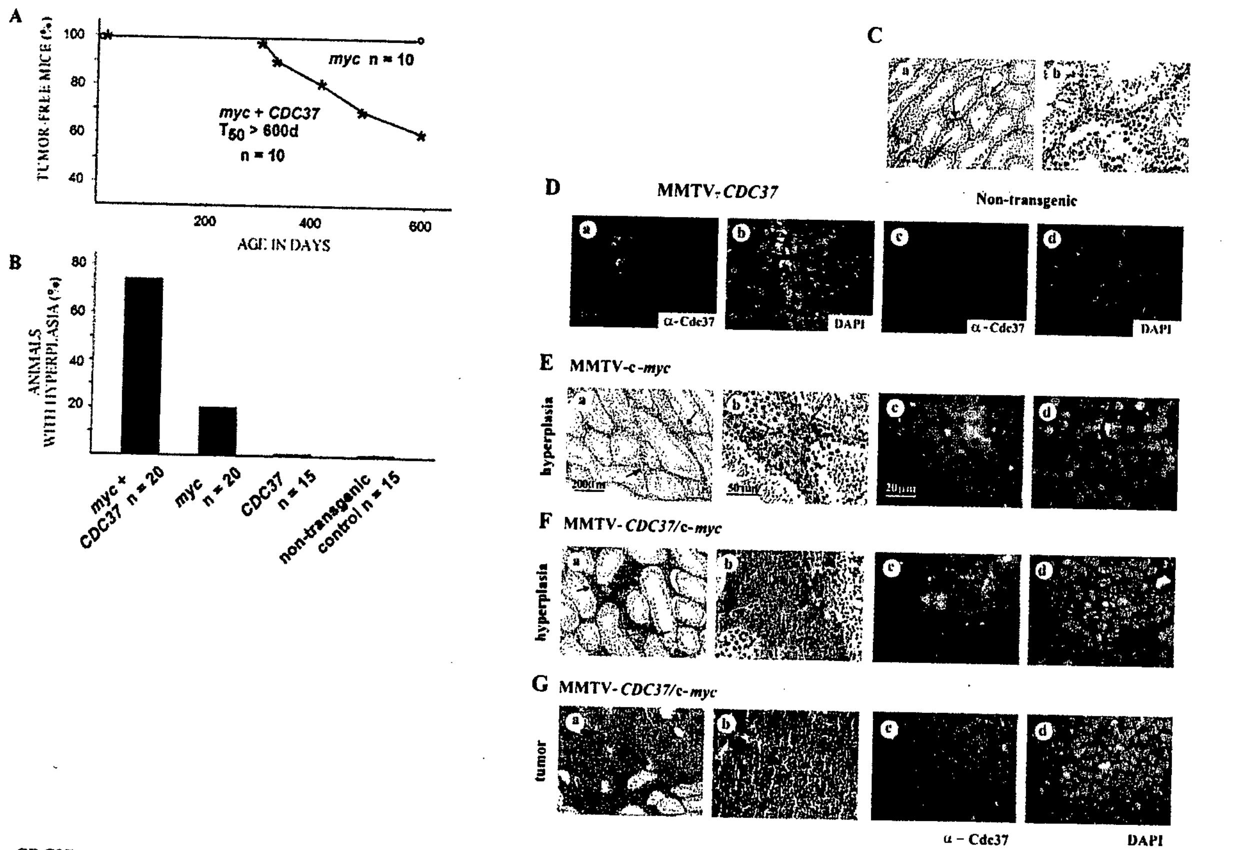


FIG. 5. *CDC37* cooperates with c-*myc* in the induction of the Leydig cell hyperplasia and transformation. (A) MMTV–*CDC37*/c-*myc* double transgenic males develop tumors, while male animals expressing a single transgene are unaffected. A plot of tumor-free mice over time is shown. Three of four tumors were Leydig cell tumors, while the fourth was a lymphoma. (B) MMTV–*CDC37*/c-*myc* double transgenic males display extensive Leydig cell hyperplasia compared to MMTV–c-*myc* and MMTV-*CDC37* animals. Histological sections of testis derived from grossly unaffected males were analyzed at 400 days of age. The number of animals in each group is shown. (C) Tissue section of a normal testis with arrows indicating the positions of Leydig cells located between seminiferous tubules with active spermatogenesis: (a) ×100 magnification, H&E staining; and (b) ×400 magnification, H&E staining to show the usual number and morphology of Leydig cells. (D) Expression of *CDC37* in the testis of MMTV-*CDC37* transgenic (a and b) or nontransgenic (c and d) male mice at ×1,000 magnification: (a and c) *CDC37* expression in the cytoplasm of Leydig cells; and (b and d) DAPI staining to identify nuclei. (E) Mild hyperplasia found in 20% of 400-day-old males expressing MMTV–c-*myc*. (F) High-grade hyperplasia found in 75% of 400-day-old MMTV–*CDC37*/c-*myc* mice. (G) Example of a Leydig cell tumor found in MMTV–*CDC37*/c-*myc* mice: (a) ×100 magnification, H&E staining; (b) ×400 magnification, H&E staining; (c) ×1,000 magnification field stained with anti-Cdc37 antibodies; and (d) ×1,000 magnification, DAPI staining of the same field as panel c to identify nuclei.

erative disturbances were found in MMTV-cyclin D1 mice for up to 650 days (Fig. 7). Similar results have been noted in other mixed genetic backgrounds with MMTV-cyclin D1 mice (E. V. Schmidt and A. Arnold, unpublished data). Animals expressing both cyclin D1 and *CDC37* display evidence of mammary tumors at the age of 13 months, at which time control MMTV-*CDC37* and MMTV-cyclin D1 mice had yet to display a transformation phenotype (Fig. 7A and B).

Tumors developed by double transgenic animals appeared as rapidly dividing single mass adenocarcinomas. The majority of adenocarcinomas were well-differentiated carcinomas with high levels of secretion, although several cases of poorly differentiated adenocarcinomas without apparent secretion were also observed (Fig. 7C). The majority of animals developed one mammary tumor, but frequent cases of metastasis to the lung was observed during pathological analysis (Fig. 7Cd).

Each of the double and single transgenic animals subjected to the detailed pathological analysis also displayed several foci of hyper- and metaplastic mammary epithelia (Fig. 7a). The appearance of hyperplastic areas was reported previously for the single MMTV-cyclin D1 transgenics (64). In our experiment, the frequency of the appearance of the hyper- and metaplastic foci was similar in MMTV-cyclin D1 single and MMTV-*CDC37*/cyclin D1 double transgenic animals at a similar age.

Inappropriate mammary duct development in male MMTV-*CDC37* mice. Phenotypic analysis of mammary glands during development failed to identify significant differences between female MMTV-*CDC37* mice and their wild-type littermates, except for a 2- to 3-day delay in the rate of involution after lactation (data not shown). However, we did observe alterations in the development of male ductal systems, as assessed by whole-mount analysis. The development of rudimentary mammary ducts begins during embryonic development. Sexual dimorphism is already pronounced at embryonic day 14 when the male anlage undergoes significant cell death caused by androgens (22, 50). The degree of breast duct development varies in different mouse strains, ranging from the presence of the initial ductal sprout in some of the fat pads to a relatively well developed branching ductal tree. In the strain background used here, male mice do not develop a significant mammary duct structure, although the fat pad is well developed. In contrast, 60 to 70% of the adult MMTV-*CDC37* male mice have well-formed breast ducts with different degrees of elaboration by the age of 7 months (Fig. 8). In the MMTV-*CDC37*.2 line,

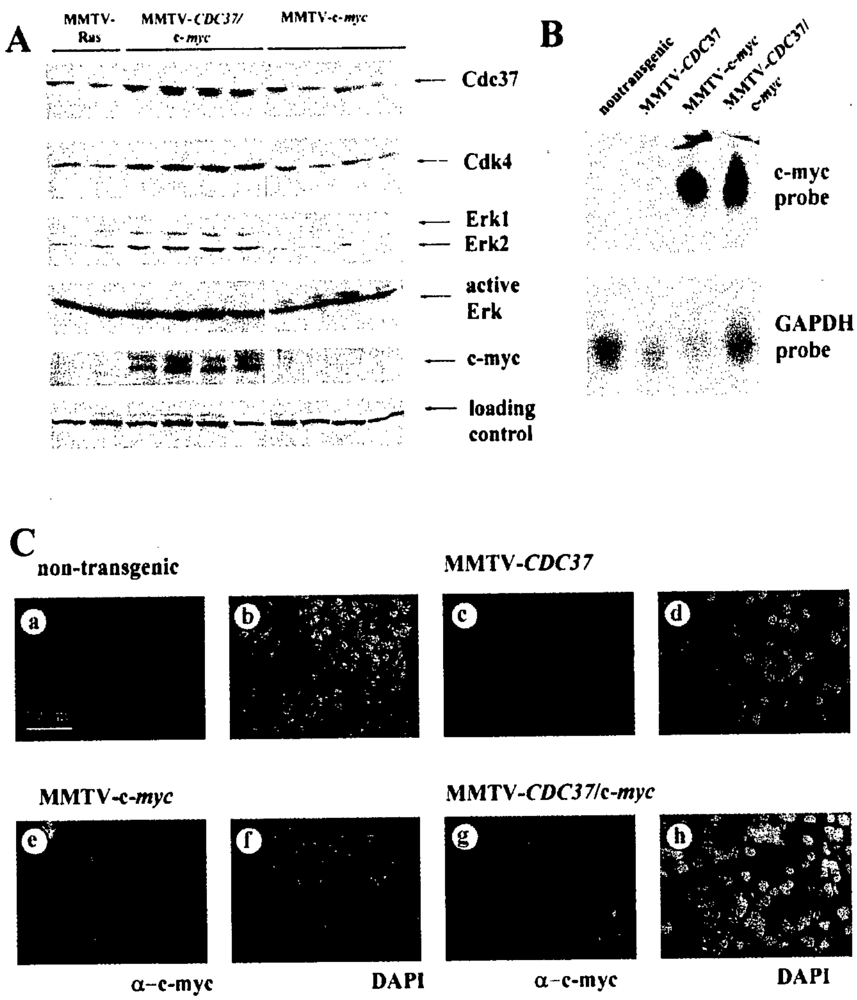


FIG. 6. MMTV–*CDC37*/c-*myc* mammary tumors have higher levels of multiple signaling proteins than tumors from MMTV–c-*myc* animals. (A) Protein extracts (200 μg/lane) from individual tumors derived from the indicated animals were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. (B) Northern blot analysis of c-*myc* mRNA in salivary tissue from nontransgenic, MMTV–c-*myc*, and MMTV–*CDC37*/c-*myc* mice. Blots were stripped and reprobed with GAPDH as a loading control. (C) Expression of c-*myc* in phenotypically normal salivary gland tissue from nontransgenic (a and b), MMTV-*CDC37* (c and d), MMTV–c-*myc* (e and f), and MMTV–*CDC37*/c-*myc* (g and h) mice. (a, c, e, and g) Anti-c-*myc*. (b, d, f, and h) DAPI used to visualize nuclei.

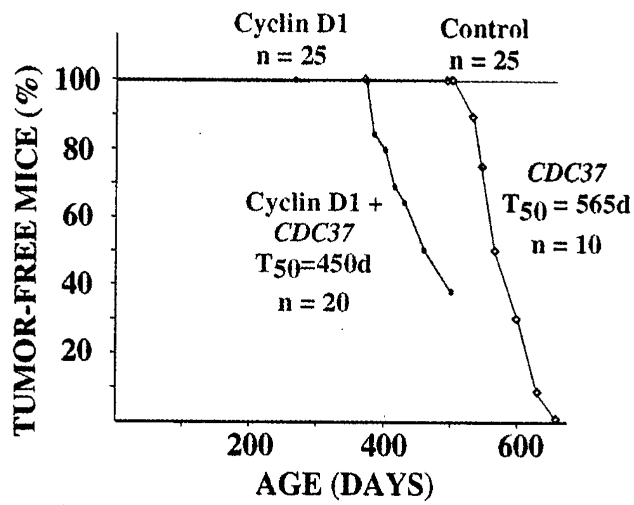
which has lower levels of expression, 30 to 40% of male animals developed breast ducts in the inguinal fat pad by the age of 7 months. In control nontransgenic littermates of the similar mixed background, only 10% of adult males have a nonbranching initial sprout structure (Fig. 8B).

To monitor the age dependence of the effect, we performed whole-mount analysis of the male mammary glands at different ages (Fig. 8B). This analysis demonstrated that 70% of 4-week-old MMTV-*CDC37*.1 and control animals have a tiny initial breast sprout which later would give rise to breast ducts. During the first 6 weeks after birth, this ductal sprout regressed in most of the nontransgenic animals, and the fraction that maintained a ductal sprout (10%) did not change for up to 8 weeks and later (Fig. 8B). In contrast, the percentage of MMTV-*CDC37* animals that maintain and elaborate ductal systems remained at ~70%. At 6 weeks of age 70% of transgenic animals have about the same or somewhat better developed initial sprout, and by 8 weeks 70% of transgenic animals have a well-developed branching duct system resembling the structures found in older MMTV-*CDC37* males. There was no significant change in breast duct development between the ages of 8 weeks and 7 months in both transgenic and control groups. The mechanism underlying this developmental alteration is not known at present but could reflect effects of *CDC37* on the androgen receptor, as has been observed in budding yeast cells (13).

DISCUSSION

Proliferation requires the coordinated activation of multiple signaling pathways, which ultimately converge on the cell cycle machinery to promote DNA replication and cell division. Studies in a variety of systems suggest that Cdc37 and Hsp90 are required to establish important signaling pathways through interaction with intrinsically unstable kinases, including the oncoprotein kinases Cdk4 and Raf-1 and *src* family members (16, 58, 62, 71). In this study, we have examined the proliferative role of *CDC37* through analysis of MMTV-*CDC37* transgenic mice. Remarkably, we found that expression of *CDC37* alone promotes neoplastic transformation of both the mammary epithelium and cells of the lymphoid compartment in older females. In this context, *CDC37* functions as a weak oncogene with rates of transformation similar to that observed previously in MMTV-cyclin D1 mice (onset at 18 to 22

A



B

Neoplasia in MMTV-cyclin D1/*CDC37* females

Mammary tumors/animals$	Tumors with metastasis (%)	Mean age at onset (days)
15/12	30	430

C

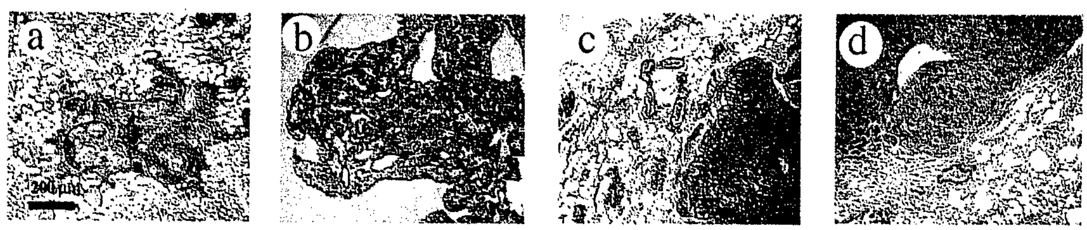


FIG. 7. Cooperation between *CDC37* and cyclin D1 oncogenes in breeding female mice. (A) By 15 months of age, a significant number of MMTV-*CDC37*/cyclin D1 double transgenic breeding females developed mammary adenocarcinomas, while none of the single transgenics developed tumors. A plot of the number of tumor-free animals over the time is shown. (B) Neoplasms developed by MMTV-cyclin D1/*CDC37* breeding females were all mammary adenocarcinomas with frequent methastasis to the lung. $, number of animals that developed palpable tumors. (C) Histological analysis of proliferative disorders (×100 magnification, H&E staining): (a) metaplastic and hyperplastic changes observed in both single MMTV-cyclin D1 and double MMTV-*CDC37*/cyclin D1 transgenic females; (b) well-differentiated secreting mammary adenocarcinoma, developed by MMTV-*CDC37*/cyclin D1 double transgenic female; (c) poorly differentiated mammary adenocarcinoma, developed by MMTV-*CDC37*/cyclin D1 female; and (d) lung metastasis from a double transgenic mouse.

months) (64). Mammary tumors from these animals displayed low mitotic activity, a finding consistent with their very slow development and growth. Two independent lines of MMTV-*CDC37* mice both displayed transformation in tissues known to be transformed by MMTV-driven oncogenes, although the penetrance of the phenotype is not as severe in the MMTV-*CDC37*.2 strain as in the MMTV-*CDC37*.1 strain (Table 1). Transgenic mice expressing cyclin E, cyclin D, and *ras* also display variability in the extent and tissue specificity of transformation (3, 32, 61, 64). This variability may reflect the site of integration and/or the levels of expression. We consider it likely that the persistent expression of *CDC37* may allow what would otherwise be silent somatic mutations occurring over time in these animals to give rise to transformation. *CDC37* appears to have multiple targets, many of which can promote proliferation in various settings. Thus, it is not clear whether the multiple transformation events we have observed in MMTV-*CDC37* mice reflect mutational activation of a single collaborating pathway or mutations in different pathways in different tumors that occur stochastically.

Because of the link between Raf-1, Cdk4, and Cdc37, we asked whether *CDC37* could cooperate with c-*myc*-dependent transformation by breeding MMTV-*CDC37* and MMTV–c-*myc* mice. In principle, stabilization and/or activation of Raf-1 by ectopic Cdc37, which has been observed in heterologous systems (15), could inappropriately activate the *ras* pathway, and this could be observed as collaboration with c-*myc* in vivo. c-*myc* can collaborate with *ras* to transform a variety of cell types both in vitro and in vivo (26, 56). The ability of *ras* to function as a growth promoter as opposed to a growth inhibitor may rely upon inactivation of the ARF/Mdm2/p53 pathway. In primary fibroblasts, *ras* can induce G_1 arrest and a senescence-like state dependent upon p53 and $p16^{INK4a}$, but this activity is lost with immortalization (29, 53). The selective pressure on c-*myc*-expressing cells to inactivate the ARF-p53 pathway or undergo apoptosis (73), therefore, provides a plausible model for collaboration between *ras* and *myc* in cellular transformation (reviewed in reference 54). c-*myc* may also promote proliferation by controlling Cdk activity. c-*myc* expression can induce Cdk4/cyclin D kinase activity in certain situations (33). There is also evidence that cyclin D1 and Cdk4 are required for the proliferative effects of c-*myc* (17, 48) and that the expression of cyclin D1 and c-*myc* could be interdependent in some systems (48). In addition, c-*myc* expression leads to cyclin E/Cdk2 kinase activation, at least in part through inactivation of p27 (28, 43, 49, 63).

We found that *CDC37* and c-*myc* collaborate to transform multiple tissues in both breeding and nonbreeding females, as well as in males, and both MMTV-*CDC37* lines behaved similarly in this regard. In breeding and virgin females, *CDC37* enhanced both the rate and extent of mammary transformation by c-*myc*. Importantly, the number of tumor foci observed with

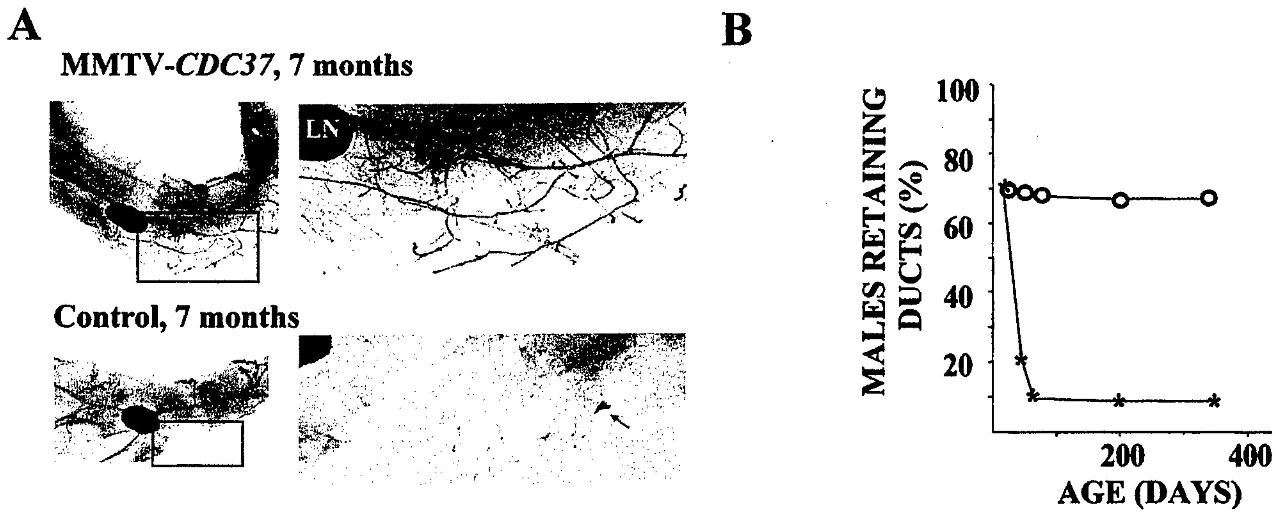


FIG. 8. Inappropriate mammary duct development in MMTV-*CDC37* transgenic males. (A) Whole-mount analysis of the mammary glands from transgenic males and nontransgenic littermates at 7 months of age. Inguinal mammary glands were fixed in formalin, cleared with acetone, and stained with hematoxylin to visualize mammary ducts. By 7 months, a significant number of transgenic males developed an extensive system of breast ducts resembling that of a normal virgin female, while only 10% of the males in the control group had retained an initial sprout. LN, lymph node; black arrow, initial duct sprout in a nontransgenic male. (B) Percentage of transgenic and nontransgenic animals retaining breast structures as a function of age. For each time point, more than 10 inguinal mammary glands were autopsied and analyzed.

c-*myc* in the presence of MMTV-*CDC37* was dramatically increased (from an average of three tumors/animal to an average approaching 20 tumors/animal) in breeding females. This result suggests that in some cell types *CDC37* expression may be rate limiting for transformation. In this regard, we have observed expression of *CDC37* in c-*myc* and *ras* induced mammary tumors, despite the fact that *CDC37* is not expressed in resting mammary epithelium. Interestingly, induction of Cdc37 is not a simple consequence of c-*myc* expression since phenotypically normal tissues expressing abundant c-*myc* lack detectable Cdc37 (Fig. 2). Thus, additional events that give rise to induction of Cdc37 are apparently occurring during the process of c-*myc*-dependent transformation. The increased rates of mammary transformation observed with pregnancy in MMTV–c-*myc* and MMTV-*ras* transgenic mice may reflect the fact that *CDC37* is normally induced during pregnancy (58) and could provide a proliferation-permissive setting that allows for these oncogenes to promote transformation. We expect that other events, including inactivation of the ARF/p53 pathway (reviewed in reference 53), are also involved in c-*myc*-mediated transformation in MMTV-*CDC37* mice.

Unexpected was the finding that Cdc37 expression allowed transformation by c-*myc* in cell types where it is normally not oncogenic. In virgin females, MMTV–*CDC37*/c-*myc* mice developed salivary tumors. Although MMTV-*ras* mice develop salivary tumors (31), MMTV–c-*myc* mice have not been reported to develop salivary tumors. The inability of c-*myc* to transform the salivary epithelium is considered a peculiarity of this oncogene. Our results suggest that the absence of *CDC37* expression in adult salivary glands may contribute to the inability of c-*myc* to transform this tissue.

We also found that expression of *CDC37* allows c-*myc* to transform Leydig cells in the testis. c-*myc* induced a very mild hyperplasia in a small fraction of the animals, but when *CDC37* was coexpressed there was a dramatic increase in the extent and severity of Leydig cell hyperplasia. Moreover, 30% of the double transgenic animals examined displayed evidence of overt Leydig cell neoplasia. The effect of *CDC37* on the extent of proliferation of Leydig cells is possibly due to its effect on Cdk4. Recent studies show that one of the phenotypes of Cdk4 knockout males is the reduction of the number of the Leydig cells and abnormalities in sperm maturation and infertility (44). Moreover, the expression of a mutant form of Cdk4 that cannot bind $p16^{INK4a}$ leads to an increased population of testicular Leydig cells (44). These studies point to the important role of Cdk4 kinase in the proliferation of this cell type. The cooperative behavior of the c-*myc* and *CDC37* in the induction of hyperproliferation and transformation in Leydig cells may therefore be explained by the role of *CDC37* in the stabilization of Cdk4 kinase (58) and c-*myc* in the induction of cyclin D1 expression (48). In contrast to c-*myc*, MMTV-*CDC37* did not affect the rate of mammary transformation induced by MMTV-*neu* in nonbreeding animals, although a severalfold increase in mitotic index was observed (data not shown).

Biochemical data indicate that Cdk4 is a major target of the Cdc37-Hsp90 chaperone complex (11, 58). If ectopic expression could inappropriately stabilize Cdk4, then one might expect to see increased proliferation in response to coexpression of cyclin D1. *CDC37* mRNA is coordinately regulated with cyclin D1 during breast development and in adult tissues, suggesting a functional link (58). Consistent with this, we found that MMTV-*CDC37* can collaborate with MMTV-cyclin D1 in the transformation of mammary epithelial cells.

Although the phenotypic consequences of *CDC37* expression and collaboration with c-*myc* and cyclin D1 are striking, the biochemical mechanisms underlying its action are likely to be complex, possibly involving multiple kinase pathways that function interdependently to promote proliferation. Stabilization and/or activation of Cdk4 or Raf could result in both activation of the *ras* pathway and activation of Cdks. In the latter case, increased Cdk4 levels could simultaneously sequester $p16^{INK4a}$ and promote proliferation via activation by cyclin D. This could, in turn, lead to activation of cyclin E-Cdk2 by both increasing cyclin E expression and by sequestration of p27. We have observed increased levels of both Cdk4 and the Erk1 kinase. Interestingly, we noticed that mammary tumors from MMTV–*CDC37*/c-*myc* animals contained significantly higher levels of c-*myc* than tumors from MMTV–c-*myc* ani-

mals independent of changes in mitotic index. This increase did not reflect effects of Cdc37 expression on MMTV-driven c-*myc* mRNA nor did it reflect an ability of Cdc37 to augment the number of cells expressing c-*myc*. Recent studies suggested that activation of the *ras* pathway stabilizes c-*myc* (51). It is therefore possible that Cdc37, through its interaction with kinases in the *ras* pathway, indirectly stabilizes c-*myc*. Since Cdc37 does not appear to have an effect on c-*myc* levels in phenotypically normal tissues, its effects on c-*myc* levels in tumors may require additional events. Further studies are required to determine whether increased levels of c-*myc* via *CDC37* expression are an important component of the collaborative effects seen in vivo.

In summary, our results suggest that the presence of Cdc37 may be rate limiting for the establishment of oncogenic signaling pathways that promote transformation. Although the effects observed here are in response to Cdc37 expression, recent studies provide correlative data indicative of a role for Cdc37 in human cancer. Normal human prostate epithelium has low to undetectable levels of Cdc37 (58a). However, Cdc37 is highly expressed in human prostatic cancer and is also expressed in preneoplastic lesions in the prostate, a finding consistent with its induction at an early stage of prostate cancer (58a). Similarly, our results indicate that Cdc37 induction occurs during transformation by c-*myc*. The mechanisms responsible for Cdc37 induction during the transformation process remain to be determined. We also note that the role of *CDC37* in transformation suggested by this work may explain the sensitivity of various tumor types to clinically relevant anzamycin derivatives (23, 39, 67, 68), which are known to bind Hsp90 and disrupt *ras*- and cyclin D-dependent signaling pathways.

ACKNOWLEDGMENTS

We thank Phil Leder for providing MMTV-*ras* mice, Norman Greenberg for access to microscope facilities, Dan Medina for advice on whole-mount analysis, and Charles Sherr for enlightening discussions.

This work was supported by NIH grants GM54137 and by the Welch Foundation (J.W.H.) and by the Baylor SPORE in Prostate Cancer (F.D.). L.S. is supported by a predoctoral training grant from the Department of Defense.

REFERENCES

1. **Aktas, H., H. Cai, and G. M. Cooper.** 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. Mol. Cell. Biol. **17:**3850–3857.
2. **Albanese, C., J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, and R. G. Pestell.** 1995. Transforming p21^{ras} mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem. **270:** 23589–23597.
3. **Bortner, D. M., and M. P. Rosenberg.** 1997. Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E. Mol. Cell. Biol. **17:**453–459.
4. **Breter, H. J., J. Ferguson, T. A. Peterson, and S. I. Reed.** 1983. Isolation and transcriptional characterization of three genes which function at start, the controlling event of the *Saccharomyces cerevisiae* cell division cycle: CDC36, CDC37, and CDC39. Mol. Cell. Biol. **3:**881–891.
5. **Brugge, J. S.** 1986. Interaction of the Rous sarcoma virus protein pp60^{src} with cellular proteins pp50 and pp90. Curr. Top. Microbiol. Immunol. **123:** 1–22.
6. **Brugge, J. S.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, pp60^{src}, with two cellular proteins. Cell **25:**363–372.
7. **Cheng, M., P. Olivier, J. A. Diehl, M. Fero, M. F. Roussel, J. M. Roberts, and C. J. Sherr.** 1999. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. **18:**1571–1583.
8. **Cheng, M., V. Sexl, C. J. Sherr, and M. F. Roussel.** 1998. Assembly of cyclin D-dependent kinase and titration of p27^{Kip1} regulated by mitogen-activated protein kinase kinase (MEK1). Proc. Natl. Acad. Sci. USA **95:**1091–1096.
9. **Connell-Crowley, L., J. W. Harper, and D. W. Goodrich.** 1997. Cyclin D1/ Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell **8:**287–301.
10. **Cutforth, T., and G. M. Rubin.** 1994. Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in *Drosophila*. Cell **77:** 1027–1036.
11. **Dai, K., R. Kobayashi, and D. Beach.** 1996. Physical interaction of mammalian CDC37 with CDK4. J. Biol. Chem. **271:**22030–22034.
12. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**487–497.
13. **Fliss, A. E., Y. Fang, F. Boschelli, and A. J. Caplan.** 1997. Differential in vivo regulation of steroid hormone receptor activation by cdc37p. Mol. Biol. Cell **8:**2501–2509.
14. **Gerber, M. R., A. Farrell, R. J. Deshaies, I. Herskowitz, and D. O. Morgan.** 1995. Cdc37 is required for association of the protein kinase Cdc28 with G1 and mitotic cyclins. Proc. Natl. Acad. Sci. USA **92:**4651–4655.
15. **Grammatikakis, N., A. Grammatikakis, M. Yoneda, Q. Yu, S. D. Banerjee, and B. P. Toole.** 1995. A novel glycosaminoglycan-binding protein is the vertebrate homologue of the cell cycle controlprotein, Cdc37. J. Biol. Chem. **270:**16198–16205.
16. **Grammatikakis, N., J. H. Lin, A. Grammatikakis, P. N. Tsichlis, and B. H. Cochran.** 1999. p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. Mol. Cell. Biol. **19:**1661–1672.
17. **Haas, K., P. Staller, C. Geisen, J. Bartek, M. Eilers, and T. Moroy.** 1997. Mutual requirement of CDK4 and Myc in malignant transformation: evidence for cyclin D1/CDK4 and p16INK4A as upstream regulators of Myc. Oncogene **15:**179–192.
18. **Hutchison, K. A., B. K. Brott, J. H. De Leon, G. H. Perdew, R. Jove, and W. B. Pratt.** 1992. Reconstitution of the multiprotein complex of pp60^{src}, hsp90, and p50 in a cell-free system. J. Biol. Chem. **267:**2902–2908.
19. **Kato, J., M. Matsuoka, K. Polyak, J. Massague, and C. J. Sherr.** 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell **79:**487–496.
20. **Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7:**331–342.
21. **Kerkhoff, E., and U. R. Rapp.** 1997. Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. Mol. Cell. Biol. **17:**2576–2586.
22. **Kratochwil, K.** 1975. Experimental analysis of the prenatal development of the mammary gland. Modern Problems Paediatr. **15:**1–15.
23. **Kwon, H. J., M. Yoshida, K. Muroya, S. Hattori, E. Nishida, Y. Fukui, T. Beppu, and S. Horinouchi.** 1995. Morphology of ras-transformed cells becomes apparently normal again with tyrosine kinase inhibitors without a decrease in the ras-GTP complex. J. Biochem. **118:**221–228.
24. **LaBaer, J., M. D. Garrett, L. F. Stevenson, J. M. Slingerland, C. Sandhu, H. S. Chou, A. Fattaey, and E. Harlow.** 1997. New functional activities for the p21 family of CDK inhibitors. Genes Dev. **11:**847–862.
25. **Lamphere, L., F. Fiore, X. Xu, L. Brizuela, S. Keezer, C. Sardet, G. F. Draetta, and J. Gyuris.** 1997. Interaction between Cdc37 and Cdk4 in human cells. Oncogene **14:**1999–2004.
26. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304:**596–602.
27. **Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur.** 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem. **271:**20608–20616.
28. **Leone, G., J. DeGregori, R. Sears, L. Jakoi, and J. R. Nevins.** 1997. Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature **387:**422–426.
29. **Lin, A. W., M. Barradas, J. C. Stone, van L. Aelst, M. Serrano, and S. W. Lowe.** 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev. **12:** 3008–3019.
30. **Lukas, J., J. Bartkova, M. Rohde, M. Strauss, and J. Bartek.** 1995. Cyclin D1 is dispensable for G_1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. **15:**2600–2611.
31. **Mangues, R., I. Seidman, A. Pellicer, and J. W. Gordon.** 1990. Tumorigenesis and male sterility in transgenic mice expressing a MMTV/N-ras oncogene. Oncogene **5:**1491–1497.
32. **Mangues, R., I. Seidman, J. W. Gordon, and A. Pellicer.** 1992. Overexpression of the N-ras proto-oncogene, not somatic mutational activation associated with malignant tumors in transgenic mice. Oncogene **7:**2073–2076.
33. **Mateyak, M. K., A. J. Obaya, and J. M. Sedivy.** 1999. c-Myc regulates cyclin D-cdk4 and -cdk6 activity but affects cell cycle progression at multiple independent points. Mol. Cell. Biol. **19:**4672–4683.
34. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J. Y. Kato.** 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. 1994 **14:**2066–2076.
35. **McConnell, B. B., F. J. Gregory, F. J. Stott, E. Hara, and G. Peters.** 1999. Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. Mol. Cell. Biol. **19:**1981–1989.
36. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for

cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14**:2077–2086.

37. **Mittnacht, S., H. Paterson, M. F. Olson, and C. J. Marshall.** 1997. Ras signalling is required for inactivation of the tumour suppressor pRb cell-cycle control protein. Curr. Biol. **7**:219–221.
38. **Munoz, M. J., and J. Jimenez.** 1999. Genetic interactions between Hsp90 and the Cdc2 mitotic machinery in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. **261**:242–250.
39. **Murakami, Y., S. Mizuno, M. Hori, and Y. Uehara.** 1988. Reversal of transformed phenotypes by herbimycin A in *src* oncogene expressed rat fibroblasts. Cancer Res. **48**:1587–1590.
40. **Parry, D., D. Mahony, K. Wills, and E. Lees.** 1999. Cyclin D-cdk subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. Mol. Cell. Biol. **19**:1775–1783.
41. **Peeper, D. S., T. M. Upton, M. H. Ladha, E. Neuman, J. Zalvide, R. Bernards, J. A. DeCaprio, and M. E. Ewen.** 1997. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature **386**:177–181.
42. **Perdew, G. H., H. Wiegand, J. P. Vanden Heuvel, C. Mitchell, and S. S. Singh.** 1997. A 50 kilodalton protein associated with raf and pp60(v-src) protein kinases is a mammalian homolog of the cell cycle control protein cdc37. Biochemistry **36**:3600–3607.
43. **Perez-Roger, I., D. L. Solomon, A. Sewing, and H. Land.** 1997. Myc activation of cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. Oncogene **14**: 2373–2381.
44. **Rane, S. G., P. Dubus, R. V. Mettus, E. J. Galbreath, G. Boden, E. P. Reddy, and M. Barbacid.** 1999. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. Nat. Genet. **22**:44–52.
45. **Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massague.** 1995. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. **9**:1831–1845.
46. **Reynisdottir, I., and J. Massague.** 1997. The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev. **11**:492–503.
47. **Robbins, D. J., M. Cheng, E. Zhen, C. A. Vanderbilt, L. A. Feig, and M. H. Cobb.** 1992. Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. Proc. Natl. Acad. Sci. USA **89**:6924–6928.
48. **Roussel, M. F., A. M. Theodoras, M. Pagano, and C. J. Sherr.** 1995. Rescue of defective mitogenic signaling by D-type cyclins. Proc. Natl. Acad. Sci. USA **92**:6837–6841.
49. **Rudolph, B., R. Saffrich, J. Zwicker, B. Henglein, R. Muller, W. Ansorge, and M. Eilers.** 1996. Activation of cyclin-dependent kinases by Myc mediates induction of cyclin A, but not apoptosis. EMBO J. **15**:3065–3076.
50. **Sakakura, T.** 1987. Mammary embryogenesis, p. 37–66. *In* M. C. Neville and C. W. Daniel (ed.), The mammary gland: development, regulation, and function. Plenum Press, New York, N.Y.
51. **Sears, R., G. Leone, J. DeGregori, and J. R. Nevins.** 1999. Ras enhances Myc protein stability. Mol. Cell **3**:169–179.
52. **Serrano, M., E. Gomez-Lahoz, R. A. DePinho, D. Beach, and D. Bar-Sagi.** 1995. Inhibition of ras-induced proliferation and cellular transformation by p16INK4. Science **267**:249–252.
53. **Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach, and S. W. Lowe.** 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell **88**:593–602.
54. **Sherr, C. J.** 1998. Tumor surveillance via the ARF-p53 pathway. Genes Dev. **12**:2984–2991.
55. **Sherr, C. J., and J. M. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9**:1149–1163.
56. **Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder.** 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell **49**:465–475.
57. **Stancato, L. F., Y.-H. Chow, K. A. Hutchison, G. H. Perdew, R. Jove, and W. B. Pratt.** 1993. Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. J. Biol. Chem. **268**:21711–21716.
58. **Stepanova, L., X. Leng, S. Parker, and J. W. Harper.** 1996. Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. Genes Dev. **10**:1491–1502.

58a. **Stepanova, L., G. Yang, F. DeMayo, T. M. Wheeler, M. Finegold, T. C. Thompson, and J. W. Harper.** Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to promote prostatic hyperplasia. Oncogene, in press.

59. **Stewart, T. A., P. K. Pattengale, and P. Leder.** 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MMTV/myc fusion genes. Cell **38**:627–637.
60. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68**:1031–1040.
61. **Tremblay, P. J., F. Pothier, T. Hoang, G. Tremblay, S. Brownstein, A. Liszauer, and P. Jolicaeur.** 1989. Transgenic mice carrying the mouse mammary tumor virus *ras* fusion gene: distinct effects in various tissues. Mol. Cell. Biol. **9**:854–869.
62. **van der Straten, A., C. Rommel, B. Dickson, and E. Hafen.** 1997. The heat shock protein 83 (Hsp83) is required for Raf-mediated signalling in *Drosophila*. EMBO J. **16**:1961–1969.
63. **Vlach, J., S. Hennecke, K. Alevizopoulos, D. Conti, and B. Amati.** 1996. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J. **15**:6595–6604.
64. **Wang, T. C., R. D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E. V. Schmidt.** 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature **369**:669–671.
65. **Warne, P. H., P. R. Viciana, and J. Downward.** 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature **364**:352–355.
66. **Whitelaw, M. L., K. Hutchison, and G. H. Perdew.** 1991. A 50-kDa cytoxolic protein complexed with the 90-kDa Heat shock protein (hsp90) is the same protein complexed with pp60$^{v\text{-}src}$ hsp90 in cells transformed by the Rous sarcoma virus. J. Biol. Chem. **266**:16436–16440.
67. **Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers.** 1994. Inhibition of heat shock protein HSP90-pp60$^{v\text{-}src}$ heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA **117**:8324–8328.
68. **Whitesell, L., S. D. Shifrin, G. Schwab, and L. M. Neckers.** 1992. Benzoquinoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition. Cancer Res. **52**:1721–1728.
69. **Winston, J. T., S. R. Coats, Y.-Z. Wang, and W. J. Pledger.** 1996. Regulation of the cell cycle machinery by oncogenic *ras*. Oncogene **12**:127–134.
70. **Wood, K. W., C. Sarnecki, J. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68**:1041–1050.
71. **Xu, Y., M. A. Singer, and S. Lindquist.** 1999. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. Proc. Natl. Acad. Sci. USA **96**:109–114.
72. **Zhang, X.-F., J. Settleman, J. M. Kyriakis, E. Takeuchi-Suzuki, S. J. Elledge, M. S. Marshall, J. T. Bruder, U. R. Rapp, and J. Avruch.** 1993. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature **364**:308–313.
73. **Zindy, F., C. M. Eischen, D. H. Randle, T. Kamijo, J. L. Cleveland, C. J. Sherr, and M. F. Roussel.** 1998. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev. **12**: 2424–2433.

Oncogene (2000) 19, 2186–2193

www.nature.com/onc

Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to promote prostatic hyperplasia

Lilia Stepanova[1,5], Guang Yang[3], Franco DeMayo[4], Thomas M Wheeler[2], Milton Finegold[2], Timothy C Thompson[3,4] and J Wade Harper*,[1]

[1]*Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, TX 77030, USA;* [2]*Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, TX 77030, USA;* [3]*Scott Department of Urology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, TX 77030, USA;* [4]*Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, TX 77030, USA*

The Cdc37 gene encodes a 50 kDa protein which targets intrinsically unstable oncoprotein kinases such as Cdk4, Raf-1, and src to the molecular chaperone Hsp90. This activity is thought to play an important role in the establishment of signaling pathways controlling cell proliferation. The budding yeast Cdc37 homolog is required for cell division and mammalian Cdc37 is expressed in proliferative zones during embryonic development and in adult tissues, consistent with a positive role in proliferation. Here we report that human prostatic tumors, neoplasias and certain pre-malignant lesions display increased Cdc37 expression, suggesting an important and early role for Cdc37 in prostatic transformation. To test the consequences of increased Cdc37 levels, transgenic mice expressing Cdc37 in the prostate were generated. These mice displayed a wide range of growth-related abnormalities including prostatic epithelial cell hyperplasia and dysplasia. These data suggest that the expression of Cdc37 may promote inappropriate proliferation and may be an important early step in the development of human prostate cancer. *Oncogene* (2000) **19,** 2186–2193.

Keywords: Cdc37; cyclin dependent kinase; chaperone; cancer; transgenic mice

Introduction

Benign prostatic hyperplasia (BPH) and prostate cancer are the most common prostate abnormalities affecting older men. These are multifactorial disease processes, which involve biochemical, genetic and epigenetic factors. Their pathogenesis, however, remains poorly understood. Nodular or glandular hyperplasia is diagnosed in about 20% of males by 40 years of age. The frequency of nodular hyperplasia is increased progressively with age, reaching more than 90% of men in their eighties (Coffey, 1992; Walsh, 1992). Prostate carcinoma is the most frequently diagnosed cancer, with incidence approaching 60% in men older than 80 years of age, and is the second most common cause of cancer-related deaths in men. Despite significant advances in understanding and treating cancer in general, the mortality resulting from prostate cancer has remained high (Landis *et al.*, 1999).

Prostate cancer is a complex disease in which focal transformation occurs with frequent and unpredictable metastasis. A variety of mutations have been linked with prostate cancer (reviewed in Thompson *et al.*, 1999) and many of these mutations impinge on some aspect of cell cycle control. The decision to enter the cell division cycle is made during G1 (first gap phase), a period where cells are responsive to extracellular growth signals. In response to net positive signals, cells activate signal transduction pathways that culminate in the expression genes required for cell cycle progression. The *ras* signaling pathway is a central component of this transduction cascade and has been shown to be required for the induction of cyclin D1 expression (Aktas *et al.*, 1997; Peeper *et al.*, 1997). D-type cyclins function as regulatory subunits of Cdk4 and Cdk6 and these complexes are required for the G1-S transition. Because the levels of D-type cyclins are sensitive to the presence of mitogens, they function as sensors to control the decision to enter S phase. Cdk4 and Cdk6 have been implicated in the inactivation of Rb, leading to activation of E2F-dependent transcriptional programs, and in titration of the Cdk2 inhibitor $p27^{KIP1}$, leading to activation of cyclin E/Cdk2 which is also required for S phase. (Ewen *et al.*, 1993; Kato *et al.*, 1993, 1994; Matsushime *et al.*, 1994; Meyerson and Harlow, 1994; Sherr and Roberts, 1999; Reynisdottir *et al.*, 1995; Connell-Crowley *et al.*, 1997; Reynisdottir and Massague, 1997; Cheng *et al.*, 1998; McConnell *et al.*, 1999). Assembly of an active Cdk4/cyclin D complex is a multistep process involving at least one mitogen-dependent step (Matsushime *et al.*, 1994; Meyerson and Harlow, 1994; LaBaer *et al.*, 1997; Cheng *et al.*, 1998, 1999). Newly synthesized Cdk4 is assembled into a Cdc37/Hsp90 chaperone complex for stabilization (Dai *et al.*, 1996; Stepanova *et al.*, 1996; Lamphere *et al.*, 1997). Stabilized Cdk4 is apparently then released in a still uncharacterized step, thereby allowing assembly with either regulatory subunits such as cyclin D or with inhibitors such as p16 (reviewed in Sherr and Roberts, 1999).

Previously, we demonstrated that the Cdc37 gene encodes the Hsp90-associated p50 protein (Stepanova *et al.*, 1996). p50 was previously seen in complexes with several kinases implicated in mitogenic signaling pathways, including v-*src* (Brugge, 1981, 1986; Whitelaw *et al.*, 1991; Hutchison *et al.*, 1992); *src* homologs (Brugge, 1986) and Raf (Stancato *et al.*, 1993), but its identity was unknown. Genetic and biochemical

*Correspondence: J Wade Harper
[5]Current address: Department of Tumor Cell Biology, St. Judes Children's Research Hospital, Memphis, Tennessee, TN 38105, USA
Received 16 November 1999; revised 2 March 2000; accepted 7 March 2000

data in several systems suggests that particular protein kinases are intrinsically unstable and their association with the Cdc37/Hsp90 chaperone in the cytoplasm is important for folding and/or activation of the targeted kinase (Cutforth and Rubin, 1994; Gerber *et al.*, 1995; Stepanova *et al.*, 1996; Grammatikakis *et al.*, 1999; Munoz and Jimenez, 1999; Xu *et al.*, 1999). Indeed, Cdc37 is an essential gene in both budding yeast and Drosophila and this is thought to reflect its role in stabilizing growth promoting kinases. Consistent with its role in promoting cell division, Cdc37 is expressed in proliferating zones during mouse development and in adult tissues but is absent from many resting cells (Stepanova *et al.*, 1996).

In this study, we have examined Cdc37 expression in normal and transformed human prostate tissues. We found that while Cdc37 protein is low or absent from normal prostatic epithelia, it is highly expressed in transformed epithelium. The presence of Cdc37 expression in some pre-malignant prostatic lesions indicates that the induction of Cdc37 expression might be an early event in the transformation process. To test the effect of the expression of Cdc37 on cell division in the prostate, we constructed two lines of transgenic mice expressing Cdc37 under control of a prostate-specific probasin promoter. As in human tissue, Cdc37 is absence from adult mouse prostatic epithelium. Interestingly, targeted expression of Cdc37 in the mouse prostate resulted in epithelial hyperplasia and dysplasia, and other growth-related abnormalities. In a parallel study, we found that MMTV-Cdc37 caused transformation of mammary epithelium and collaborated with c-*myc* and cyclin D1 to transform multiple tissues (Stepanova *et al.*, 2000). These data are consistent with a positive role for Cdc37 in promoting not only normal proliferation but also inappropriate proliferation such as that involved in transformation, and suggests that in some contexts, Cdc37 may be rate-limiting for transformation.

Results

Cdc37 is expressed in premalignant and malignant human prostate epithelia

The expression of Cdc37 mRNA in the developing mouse embryo is tightly correlated with proliferative zones, and is coincident with the cyclin D1 expression

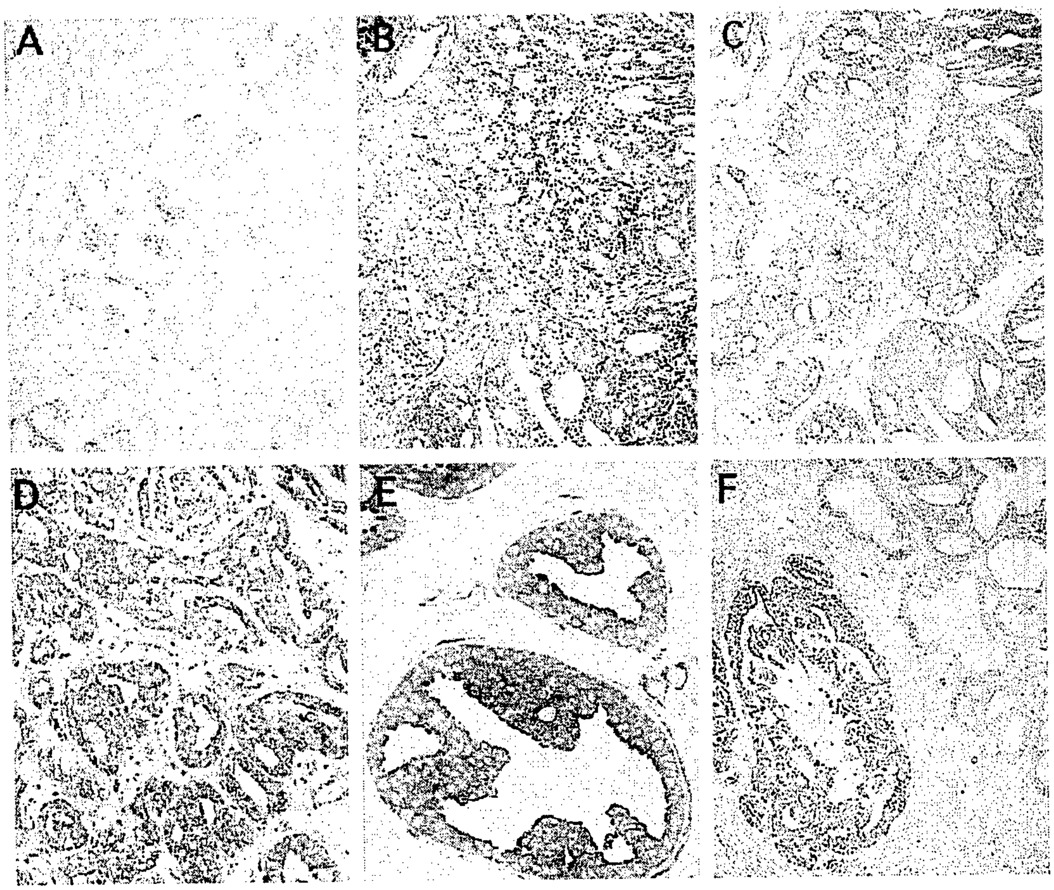


Figure 1 Cdc37 is expressed in the transformed epithelia of human prostate. (**a**) A section from a normal prostate was stained with affinity purified anti-Cdc37 antibodies. Normal epithelial and stromal compartments were typically negative for Cdc37 protein. (**b** and **c**) Adjacent sections of human prostates removed via radical prostatectomy were stained with either (B) H&E or with (**c**) anti-Cdc37 antibodies. Focal cancer are shown and Cdc37 expression is clearly observed in cancer foci. (**d**) High-magnification image of prostate cancer stained for Cdc37 expression. Cdc37 is present in the cytoplasm of a large fraction of epithelial cells in the cancer. (**e**) Cdc37 expression in a PIN-like lesion. Original magnification: **a**–**c** 100×; **d**, 200×; **e**, 400×. (**f**) A section of human prostate containing a transformed foci adjacent to largely normal galandular epithelium stained with anti-Cdc37 antibodies (100× magnification)

in certain tissues (Stepanova *et al.*, 1996). Moreover, expression of Cdc37 in the mouse breast epithelium via the MMTV promoter results in development of mammary adenocarcinomas (Stepanova *et al.*, 2000). These observations are consistent with the proposed role of Cdc37 in the establishment and maintenance of the growth promoting pathways in normal and transformed tissues.

To evaluate Cdc37 expression in the human prostate in normal and transformed states, we analysed tissue sections for Cdc37 expression using affinity purified rabbit polyclonal antibodies (Stepanova *et al.*, 1996). These antibodies recognize a 50 kDa protein in immunoblots of mouse and human fibroblast extracts and immunoprecipitate Cdc37/Cdk4/Hsp90 complexes (Stepanova *et al.*, 1996). These antibodies also specifically stain the cytoplasm of fibroblasts where Cdc37 is co-localized with Hsp90. Although weak anti-Cdc37 immunoreactivity was seen in a small proportion of glandular cells and in rare basal cells, most cells in normal prostate did not contain detectable Cdc37 protein when compared to control sections probed with normal rabbit serum (Figure 1a and data not shown). In contrast, elevated Cdc37 levels were found both in cancer cells as well in some pre-malignant lesions (Figure 1b–e). The increased expression of Cdc37 in transformed tissue is readily seen in sections where normal tissue lies adjacent to malignant foci (Figure 1f). To validate the results of immunohistochemistry, immunoblotting of normal and transformed human prostate tissue was performed. Levels of Cdc37 were low in normal tissues (Figure 2a, lanes 3–5). In contrast, levels of Cdc37 in multiple tumors were significantly higher than in normal tissues (lanes 6–8) and were comparable to levels found in human prostate cancer cell lines (DU145 and PC3) (lanes 1 and 2).

All 31 prostate carcinomas analysed exhibited remarkably elevated Cdc37 immunoreactivity, although the proportion of the cells expressing Cdc37 varied among tumors from less than 25% to almost 100% (Figures 1d and 2b). On average, about half of the tumors exhibited high levels of Cdc37 in greater than 50% of tumor cells. Quantitatively, the average Cdc37 staining score in cancers ($n=31$) was significantly higher than in the histologically normal prostate specimens ($n=15$, $P=0.0005$, Mann–Whitney test). Interestingly, however, we did not find a significant correlation between Cdc37 staining and the Gleason scores ($P=0.54$, Kruskal–Wallis test) (Figure 2b). Instead, the highest percentages of Cdc37-positive cells was found in moderately differentiated (Gleason score = 5–7) prostate cancer cells.

We analysed Cdc37 expression in pre-malignant lesion. Among the six high grade prostatic intraepithe-

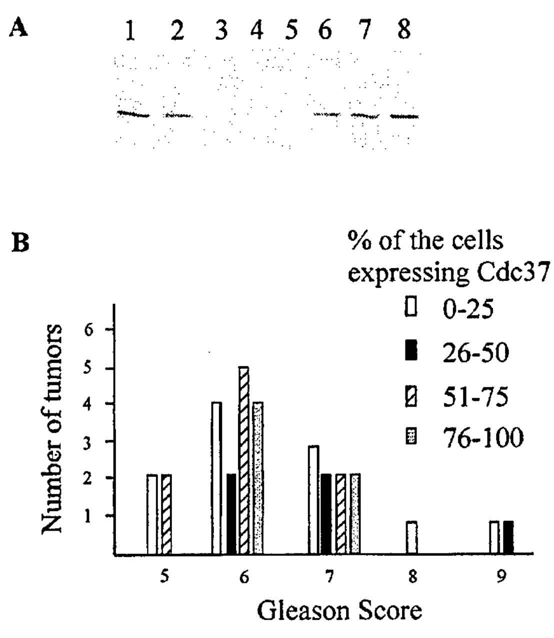


Figure 2 (a) Immunoblot analysis of Cdc37 in normal and transformed prostate tissue extracts. Lysates (80 μg) from DU145 cells (lane 1), PC3 cells (lane 2), three independent samples from normal prostate (lanes 3–5) or three independent prostate cancers (lanes 6–8) were separated by SDS–PAGE and immunoblots probed with anti-Cdc37 antibodies. Detection was accomplished using enhanced chemiluminescence. (b) The level of Cdc37 expression does not correlate with the Gleason scores of the tumors. Bars represent number of tumors expressing differential levels of Cdc37 for each of the observed values of Gleason scores. Normal tissues and BPH had staining scores of 1

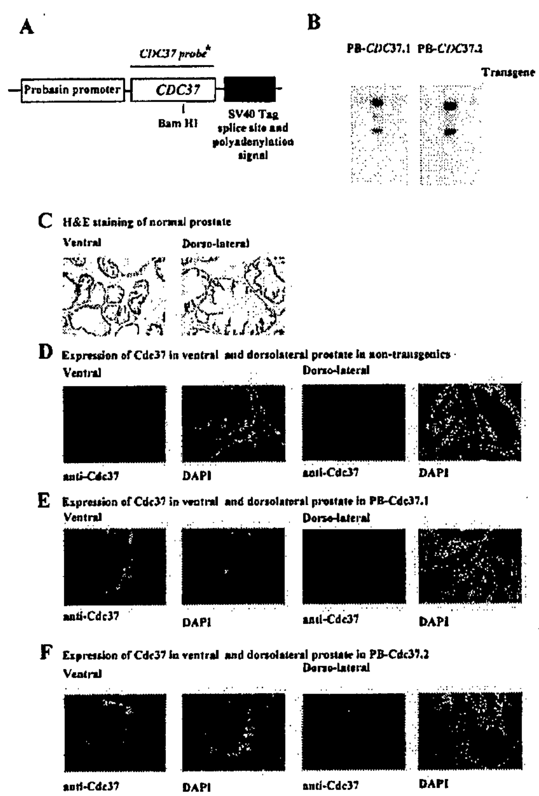


Figure 3 Characterization of PB-Cdc37 transgene expression in the normal prostate of transgenic mice. (a) Structure of the construct used to generate PB-Cdc37 mice (see Materials and methods for details). (b) Southern blot analysis of PB-Cdc37.1 and PB-Cdc37.2 transgenic lines. Tail DNA was digested with *Bam*HI prior to Southern analysis with a ^{32}P-labeled Cdc37 cDNA probe. (c–e) Expression of Cdc37 in the prostate. Sections from control and Cdc37 transgenic mice were subjected to immunofluorescence using anti-Cdc37 antibodies and nuclei were stained with DAPI. Sections from both ventral and dorso-lateral regions are shown. (d) specimens from non-transgenic animal, (e) specimens from PB-Cdc37.1 animal, (f) specimens from PB-Cdc37.2 animal

lial neoplasms (PIN) examined, five showed a remarkably high level of Cdc37 expression (Figure 1e). Occasionally, some prostatic epithelia that were adjacent to PIN or cancer cells and had undergone dysplastic changes also displayed Cdc37 immunoreactivity as well (data not shown). The presence of high levels of Cdc37 in these lesions suggest that induction of Cdc37 expression may be an early step in transformation process.

In marked contrast with tumors, Cdc37 levels in human BPH specimens were low in both stromal and epithelial compartments, with weak staining confined to focal fragments of glandular epithelia with hyperplastic features (data not shown). The average Cdc37 staining score in prostatic epithelia of BPH ($n=8$) was very close to that seen in normal prostatic epithelia ($n=15$) and significantly lower than that in cancer ($n=31$, $P=0.001$, Mann–Whitney test).

PB-Cdc37 transgenic mice

The expression of Cdc37 in early prostatic lesions suggests a possible role in prostatic transformation. To assess the significance of Cdc37 expression in promoting proliferative disorders in the prostate, transgenic mice expressing the mouse Cdc37 cDNA under control

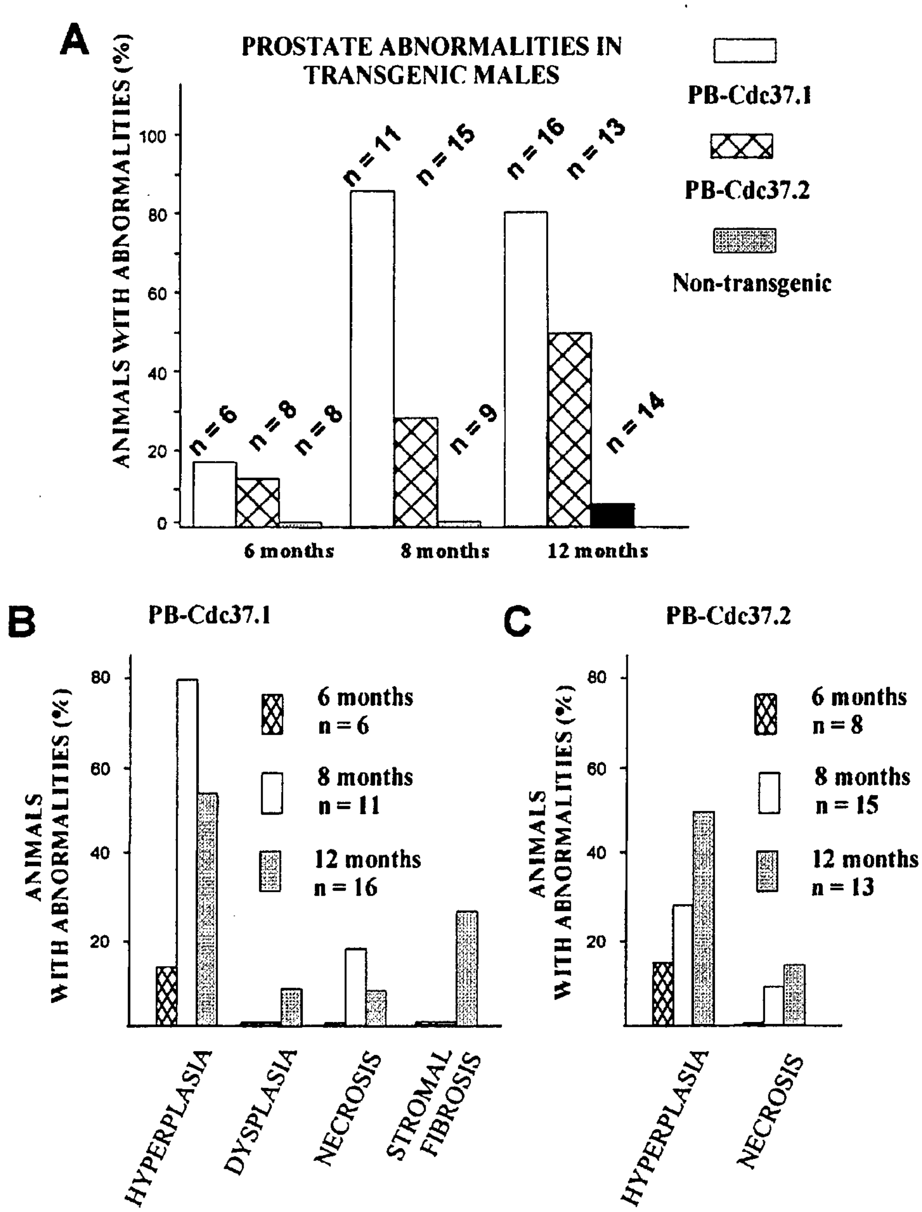


Figure 4 Morphological abnormalities in the prostate of PB-Cdc37 transgenic animals. (**a**) Quantitation of incidence of proliferative disorders at different ages. The percentage of animals with proliferative abnormalities is shown at different ages for control and transgenic males from PB-Cdc37.1 and PB-Cdc37.2 lines. Control animals used in the experiment are non-transgenic littermates of PB-Cdc37.2 males. (**b**) The observed prostatic phenotypes in PB-Cdc37.1 line at different ages. The percentage of the animals developing each phenotype is shown. Some of the animals developed more than one phenotype. (**c**) The observed prostatic phenotypes in PB-Cdc37.2 line at different ages. The percentage of the animals developing each phenotype is shown

of probasin (PB) promoter were generated (Figure 3a). The PB promoter has been shown previously to direct expression of transgenes uniquely to the prostate (Greenberg et al., 1995). Two transgenic founders were produced that transmitted the transgene to their progeny and contained multiple copies of the transgene (Figure 3b). Lines of transgenic animals (PB-Cdc37.1 and PB-Cdc37.2) were established by mating each outbred B6D2F1 × ICR founder with outbred ICR mice. The insertion of the transgenic array in the PB-Cdc37.1 line apparently occurred in the Y chromosome, since all male progeny derived from this founder were transgenic while all females were non-transgenic (data not shown). Non-transgenic littermates of the PB-Cdc37.2 line were used as controls for both lines of transgenic animals.

Expression of the Cdc37 transgene was characterized by immunofluorescence of various organs and found, as expected, to be restricted to the male prostate in both lines. Consistent with the results in humans, Cdc37 immunoreactivity was not found in normal mouse prostate epithelium (Figure 3c). In the PB-Cdc37.1 transgenic line, expression was limited to the ventral lobe of the prostate, while both ventral and lateral prostate expressed somewhat lower levels of Cdc37 in the PB-Cdc37.2 line (Figure 3c–e). On average, about 20% of the cells in ventral prostate epithelia of transgenic males in both lines showed the strong cytoplasmic staining for Cdc37, while less than 5% of cell in the dorso-lateral prostate in PB-Cdc37.2 line showed weak expression of Cdc37. Based on immunofluorescence intensity, the level of Cdc37 expression in the ventral prostate epithelium was comparable to the level of Cdc37 observed in tissue culture fibroblasts (data not shown).

Cdc37 expression causes hyperplasia in prostatic epithelium

PB-Cdc37 lines and control littermates were maintained as colonies and monitored for developmental and transformation phenotypes for more than 1 year. Transgenic animals appeared normal during the observation period. Due to the difficulty of observing prostate abnormalities in intact animals, males were sacrificed at 6, 8 and 12 months, and excised prostates examined for proliferative disorders. Growth-related abnormalities in the prostate of rodents are not frequent and their incidence increases with age. The percentage of animals acquiring growth-related abnormalities such as hyperplasia or tumors is strain dependent. On the mixed B6D2F1 × ICR genetic background used for our experiments, less than 10% of males developed some degree of hyperplasia at 12 months of age (Figure 4a). In contrast, prostatic abnormalities in male PB-Cdc37 mice could be seen as early as at 6 months of age (Figure 4a). The number of animals developing prostatic growth-related disturbances increased with age; the percentage of animals developing growth-related abnormalities increased from 18% at 6 months to more than 80% at 8 months of age in the PB-Cdc37.1 transgenic line, and from ~15% at 6 months to ~50% at 12 months of age in the PB-Cdc37.2 line (Figure 4a). The lower proportion of affected animals in the second line may reflect the lower level of Cdc37 expression in that line. All of the abnormalities in the PB-Cdc37.1 line were restricted to the ventral prostate, while both ventral and dorso-lateral prostate was affected in PB-Cdc37.2 line in roughly equal proportion despite the difference in the expression levels between ventral and dorso-lateral lobes (Figure 4 and data not shown).

The most prevalent phenotype observed in both lines was epithelial hyperplasia, which occurred in majority of PB-Cdc37.1 mice and in all PB-Cdc37.2 affected animals (Figure 4b,c). Hyperplasia was frequently accompanied by necrosis. Hyperplasia of the ventral prostate in the PB-Cdc37.1 line was observed as focal lesions involving multiple adjacent acini. Typically, >50% of acini were affected in this strain. The lesions consisted of epithelial proliferation that followed the acinar lining and did not obliterate the lumen (Figure 5a–d). Gradual transitions from completely normal to hyperplastic epithelium were observed (data not shown). Hyperplastic cells retained secretional activity, although usually it was decreased, and the cytoplasmic/nuclear ratio of the cells was

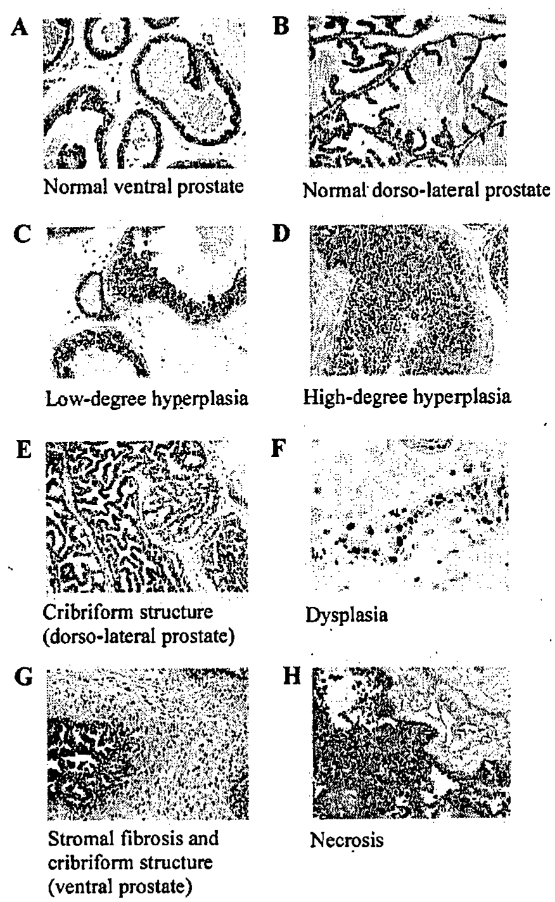


Figure 5 Phenotypes observed in the prostate of PB-Cdc37 transgenic animals. H&E staining of the sections. (**a**) Normal ventral prostate, (**b**) Normal dorso-lateral prostate, (**c**) Low-degree hyperplasia in the ventral prostate of PB-Cdc37.1 male, (**d**) High-degree hyperplasia in the ventral prostate of PB-Cdc37.1 male, (**e**) Cribriform structures formed in the dorso-lateral prostate of PB-Cdc37.2 male, (**f**) Dysplasia in the ventral prostate of PB-Cdc37.1 male, (**g**) Stromal fibrosis in the ventral prostate of PB-Cdc37.1 male adjacent to cribriform structure and (**h**) Necrosis in the ventral prostate of PB-Cdc37.1 male

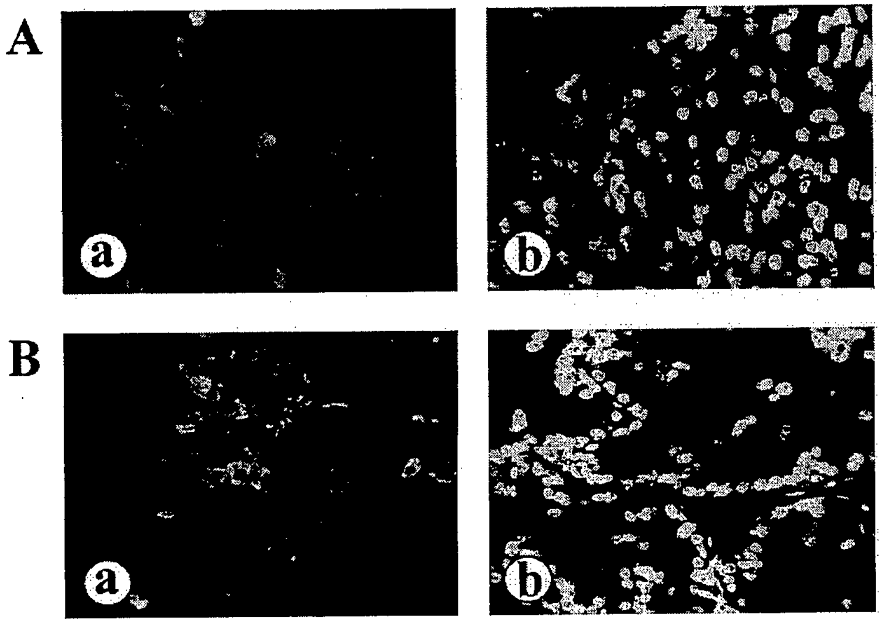


Figure 6 Cdc37 expression in the hyperplastic regions of the transgenic prostate. (a–b) Cdc37 staining (a) and DAPI nuclear staining (b) in the same section. (a) Hyperplasia. (b) Cribriform structure

increased in comparison with the normal epithelium. No inflammation was observed in the affected tissues. The development of cribriform structures such as that shown in Figure 5g, adjacent to an area of stromal fibrosis was rare. Cells in hyperplastic areas displayed minimal cellular and nuclear polymorphism. Dysplastic changes were observed in older PB-Cdc37.1 males (Figures 4b and 5f). These changes were focal, affecting usually only several adjacent acini. Single-layered epithelial cells from these regions had irregularly shaped and enlarged nuclei but no mitotic figures were observed. Stromal fibrosis was observed around the areas of high degree hyperplasia, and necrosis was observed in the ventral prostate of the oldest PB-Cdc37.1 males (Figures 4b and 5g,h).

PB-Cdc37.2 males expressed lower levels of Cdc37 in the ventral prostate, and even less in the dorso-lateral lobes. Despite the difference in expression levels between the ventral and dorsal-lateral lobes in this line, the appearance of hyperplasia was observed with equal frequency in these lobes. In both lobes, the most frequent type of the hyperplastic lesions involved cribriform structures (Figure 5e) that completely obliterated the alveolar lumen, although non-cribriform hyperplasia such as observed in the PB-Cdc37.1 line was sometimes observed in the ventral, but not dorso-lateral, prostate. Cribriform hyperplasia was typically multi-focal, affecting several adjacent alveoli. Secretion in affected alveoli was absent, in contrast with normal alveoli, and some necrosis was present in about 30% of the prostates of affected males (Figure 4c).

In both lines of transgenic animals, the abnormalities were observed only in the lobes of the prostate where Cdc37 expression was detected by immunohistochemistry (Figure 3), which correlates with the phenotype being a direct consequence of Cdc37 expression. In addition, Cdc37 expression was found throughout the affected tissue by immunofluorescence (Figure 6).

Discussion

Proliferation in a multicellular organism is a complex process requiring coordination of multiple signaling pathways that link extracellular environmental signals with the cell cycle machinery. Recent studies suggest that Cdc37/Hsp90 complexes are required for the establishment and maintenance of the signaling pathways involving kinases such as *src*, Raf-1 and Cdk4. The interaction of these intrinsically unstable kinases with the Cdc37/Hsp90 complex is required for kinase stabilization and/or activation. Since Cdc37 is required for proliferation in the variety of experimental systems, we examined Cdc37 expression in normal human prostate and in prostate cancer. Cdc37 was found to be largely absent from normal prostate epithelium but was highly expressed in all prostate tumor specimens examined, supporting the idea that Cdc37 plays an important role in proliferation. Interestingly, the highest percentages of Cdc37-positive cells were detected in moderately differentiated (Gleason score 5–7) prostate cancer. Moreover, Cdc37 was highly expressed in the majority of high-grade prostate intraepithelial neoplasias (PIN) (Figures 1 and 2). These observations suggest that the expression of Cdc37 could be an early event in the process of prostatic transformation. These results may mean that proliferative functions are selected for to a greater extent in relatively early disease, whereas prostate cancer cells, with greater metastatic potential, select for other functional activities (Bangma *et al.*, 1999). Further studies are required to elucidate the mechanisms by which Cdc37 expression is inappropriately induced during transformation.

To examine the consequences of Cdc37 expression in the prostate, we generated transgenic mice expressing Cdc37 in the prostate epithelium (Figure 3). The majority of males in two independently established transgenic lines displayed dramatic proliferative disorders in the prostate, including epithelial hyperplasia

and dysplasia not observed in control animals (Figures 4–6). Although we cannot absolutely rule out a contribution of other events in the phenotypes observed, the finding that >50% of individual acini in PB-Cdc37 animals are affected suggests that the observed phenotypes are largely, if not exclusively, a result of Cdc37 expression. The rodent prostate was previously noted for its remarkable resistance to overt transformation, and several known single oncogenes are unable to transform the prostate in transgenic models (Sharma and Schreiber-Agus, 1999). There are exceptions to this, however, as polyoma middle T and SV40 large T antigen, both of which have multiple cellular targets, give rise to prostatic transformation in transgenic animals (Sharma and Schreiber-Agus, 1999). In contrast, recent experiments have shown that MMTV-Cdc37 can lead to overt transformation in the mammary gland, although long latency (~20 months) suggests that additional genetic events are important for transformation (Stepanova *et al.*, 2000). Interestingly, the extent and rate of transformation in MMTV-Cdc37 animals were greatly enhanced in mice that also expressed either c-*myc* or cyclin D1. In the case of c-*myc*, collaboration with Cdc37 was also observed in the salivary gland and in the Leydig cells of the testis. It will, therefore, be important to determine whether other oncogenes can collaborate with Cdc37 to transform the prostate.

Although the phenotypic consequences of Cdc37 expression in the prostate are striking, the biochemical mechanisms underlying its action are likely to be complex, possibly involving multiple kinase pathways that function interdependently to promote proliferation and possibly other activities consistent with malignancy. Stabilization and/or activation of Cdk4 or Raf could result in both activation of the *ras* pathway and activation of Cdks. In the later case, increased Cdk4 levels could simultaneously sequester p16^{INK4a} and promote proliferation via activation by cyclin D1. This could, in turn, lead to activation of cyclin E/Cdk2 by both increasing cyclin E expression and by sequestration of p27. Interestingly, previous studies have found evidence for increased levels of D-type cyclins in prostate cancer (Aaltomaa *et al.*, 1999; Bubendorf *et al.*, 1999; Han *et al.*, 1998; Kallakury *et al.*, 1997) and the available data would indicate that induction of Cdc37 in tumor cells is required to support Cdk4 stability and facilitate formation of active Cdk4/cyclin D complexes that promote proliferation. The induction of components of the cyclin D pathway in prostate cancer is consistent with the low frequency of loss of the Rb tumor suppressor pathway in prostate cancer (Tamboli *et al.*, 1998).

Materials and methods

Histology and immunohistochemistry

Mouse prostates from both PB-cdc37 transgenic and normal animals were excised, fixed in 4% formaldehyde in PBS overnight at 4°C, and embedded in paraffin. Sections (5 μm) were made and stained with hematoxylin and eosin (H&E) for histological evaluation. Human prostate specimens including 31 adenocarcinomas, eight benign prostatic hyperplasias (BPH), eight intraepithelial neoplasia (PIN, high grade, $n=6$, low grade, $n=2$) as well as 15 histologically normal prostates from cystoprostatectomies or from organ donors were obtained from the SPORE tissue bank, Baylor College of Medicine, Houston. The specimens were fixed in 10% formalin, paraffin-embedded, and cut into 6 μm sections. Pathological diagnoses, histological evaluation and cancer Gleason score were made on H&E-stained sections by a single pathologist (TM Wheeler).

Cdc37 immunostaining in fixed tissues were performed with rabbit polyclonal affinity purified Cdc37 antibodies as described previously (Stepanova *et al.*, 1996). Cdc37 expression in mouse tissues was visualized using FITC-conjugated secondary antibodies. For human prostate specimens, Cdc37 immunostaining was done with the same antibody in conjunction with avidin-biotin-peroxidase (ABC) kit (Vector Lab, CA, USA). Immunoreaction products were visualized using a 3,3′-diaminobenzidine/H_2O_2 solution. Cdc37 immunoreactivity in human prostate specimens was scored at 400× using a Zeiss microscope by counting Cdc37-positive cells. The Cdc37 staining scores ranged from 0: (negative staining); 1+: 0–25%; 2+: 26–50%; 3+: >51–75% to 4+: >75% of cancer cells labeled by the Cdc37 antibody.

Statistical analysis

The correlation of Cdc37 immunoreactivity with cancer Gleason score was determined by Kruskal–Wallis test. Comparisons in Cdc37 staining scores between cancer and normal prostatic or PBH epithelia were made using the Mann–Whitney test. A P value less than 0.05 was considered statistically significant.

Generation of transgenic mice

A PB-Cdc37 transgene was generated by cloning a *Xho*I fragment containing 1.6 kb of the mouse Cdc37 open reading frame (ORF) into a plasmid containing the PB promoter and SV40 splice and polyadenylation sequences. The promoter-transgene cassette was released from vector by digesting with *BssH*II and purified. Transgene DNA was microinjected into male pronuclei of B6D2F1 mouse embryos in the Baylor College of Medicine transgenic core facility. Resulting pups were screened by Southern analysis of genomic DNA isolated from mice tails and digested with *Bam*HI. To establish lines of transgenic mice, founders were continuously mated with ICR mice. All male progeny in the line PB-Cd37.1 were transgenic, while females were not, indicating possible transgene integration into the Y chromosome. Due to the absence of non-transgenic male littermates in PB-Cdc37.1 line, non transgenic littermates of heterozygous PB-Cdc37.2 parents were used as controls for both lines. Phenotypic and histological analyses were performed as described above.

Immunoblotting

Proteins from the indicated samples were extracted and 80 μg of total proteins subjected to electrophoresis on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and blotted with affinity purified anti-Cdc37 antibodies. Detection was accomplished using avidin-biotin peroxidase complex procedure. For cancer tissue, samples were selected such that cancerous tissue made up at least 70% of the tissue sample.

Acknowledgments
We thank Norman Greenberg for access to microscope facilities. This work was supported by grants from the NIH (GM54137) and the Welch Foundation to JW Harper, and by the Baylor SPORE in Prostate Cancer (NCI P50-58204) to TC Thompson. L Stepanova is supported by a Pre-Doctoral training grant from the Department of Defense.

References

Aaltomaa S, Eskelinen M and Lipponen P. (1999). *Prostate*, **38**, 175–182.
Aktas H, Cai H and Cooper GM. (1997). *Mol. Cell. Biol.*, **17**, 3850–3857.
Bangma CH, Nasu Y, Ren C and Thompson TC. (1999). *Semin. Oncol.*, **26**, 422–427.
Brugge JS. (1986). *Curr. Topics. Microb. Immunol.*, **123**, 1–22.
Brugge J. (1981). *Cell*, **25**, 363–372.
Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G and Kallioniemi OP. (1999). *Cancer Res.*, **59**, 803–806.
Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM and Sherr CJ. (1999). *EMBO J.*, **18**, 1571–1583.
Cheng M, Sexl V, Sherr CJ and Roussel MF. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 1091–1096.
Coffey DS. (1992). *Campbell's Urology*, Vol 1, 6th ed. Walsh PC, Retik AB, Stamey TA and Vaughan, Jr ED (eds). WB Saunders Co.: Philadelphia, pp. 221–266.
Connell-Crowley L, Harper JW and Goodrich DW. (1997). *Mol. Biol. Cell*, **8**, 287–301.
Cutforth T and Rubin GM. (1994). *Cell*, **77**, 1027–1036.
Dai K, Kobayashi R and Beach D. (1996). *J. Biol. Chem.*, **271**, 22030–22034.
Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J and Livingston DM. (1993). *Cell*, **73**, 487–497.
Gerber MR, Farrell A, Deshaies RJ, Herskowitz I and Morgan DO. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4651–4655.
Grammatikakis N, Lin JH, Grammatikakis A, Tsichlis PN and Cochran BH. (1999). *Mol. Cell. Biol.*, **19**, 1661–1672.
Greenberg NM, DeMayo FJ, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ and Rosen JM. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 3439–3443.
Han EK, Lim JT, Arber N, Rubin MA, Xing WQ and Weinstein IB. (1998). *Prostate*, **35**, 95–101.
Hutchison KA, Brott BK, De Leon JH, Perdew GH, Jove R and Pratt WB. (1992). *J. Biol. Chem.*, **267**, 2902–2908.
Kallakury BV, Sheehan CE, Ambros RA, Fisher HA, Kaufman Jr RP and Ross JS. (1997). *Cancer*, **80**, 753–763.
Kato J, Matsuoka M, Polyak K, Massague J and Sherr CJ. (1994). *Cell*, **79**, 487–496.
Kato J, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ. (1993). *Genes Dev.*, **7**, 331–342.
LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A and Harlow E. (1997). *Genes Dev.*, **11**, 847–862.
Lamphere L, Fiore F, Xu X, Brizuela L, Keezer S, Sardet C, Draetta GF and Gyuris J. (1997). *Oncogene*, **14**, 1999–2004.
Landis SH, Murray T, Bolden S, *et al.* (1999). *CA Cancer J. Clin.*, **49**, 8–31.
Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ and Kato JY. (1994). *Mol. Cell. Biol.*, **14**, 2066–2076.
McConnell BB, Gregory FJ, Stott FJ, Hara E and Peters G. (1999). *Mol. Cell. Biol.*, **19**, 1981–1989.
Meyerson M and Harlow E. (1994). *Mol. Cell. Biol.*, **14**, 2077–2086.
Munoz MJ and Jimenez J. (1999). *Mol. Gen. Genet.*, **261**, 242–250.
Peeper DS, Upton TM, Ladha MH, Neuman E, Zalvide J, Bernards R, DeCaprio JA and Ewen ME. (1997). *Nature*, **386**, 177–181.
Reynisdottir I, Polyak K, Iavarone A and Massague J. (1995). *Genes Dev.*, **9**, 1831–1845.
Reynisdottir I and Massague J. (1997). *Genes Dev.*, **11**, 492–503.
Sharma P and Schreiber-Agus N. (1999). *Oncogene*, **18**, 5349–5355.
Sherr CJ and Roberts JM. (1999). *Genes Dev.*, **13**, 1501–1512.
Stancato LF, Chow Y-H, Hutchison KA, Perdew GH, Jove R and Pratt WB. (1993). *J. Biol. Chem.*, **268**, 21711–21716.
Stepanova L, Leng X, Parker S and Harper JW. (1996). *Genes Dev.*, **10**, 1491–1502.
Stepanova L, Finegold M, DeMayo F, Schmidt EV and Harper JW. (2000). *Mol. Cell. Biol.*, in press.
Thompson TC, Timme TL, Bangma CH, Nasu Y, Hull GW, Hall SJ and Stapleton AMF. (1999). *Comprehensive Textbook of Genitourinary Oncology*. Coffey DS, Scardino PT, Shipley WU and Vogelzang NJ (eds). Williams & Wilkins: Baltimore, Maryland.
Tamboli P, Amin MB, Xu HJ and Linden MD. (1998). *Mod. Pathol.*, **11**, 247–252.
Walsh PC. (1992). *Campbell's Urology*, Vol 1, 6th ed. Walsh PC, Retik AB, Stamey TA and Vaughan Jr, ED (eds). WB Saunders Co.: Philadelphia, pp 1007–1022.
Whitelaw ML, Hutchison K and Perdew GH. (1991). *J. Biol. Chem.*, **266**, 16436–16440.
Xu Y, Singer MA and Lindquist S. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 109–114.